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Faculty of Science

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Doctoral Thesis

Searching for regulators of the mammalian dNTP pool

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TABLE OF CONTENT

ABBREVIATIONS

4-OHT	4-hydroxy-tamoxyfen
AM(D; T)P	Adenosine mono(di-; tri-)phosphate
APC	Anaphase-promoting complex
a-site	Activity site
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3 related
A.U.	Arbitrary units
BCL2	B-cell lymphoma 2
CDK	Cyclin dependent kinase
CHK	Checkpoint kinase
CM(D; T)P	Cytidine mono(di-; tri-)phosphate
Crt1	Compromised recognition of TCV 1
CTD	C-terminal domain
DAPI	4',6'-Diamine-2phenylindol
DDR	DNA damage response
DNP	*De novo* pathway
(d)NTP	(desoxy) Nucleotide

dpc	Days post coitum
DSB	Double strand break
E2F	E2F transcription factor
γH2AX	Phosphorylated form of histone H2AX (S139)
G0, G1, G2, S	Cell cycle phases; Gap 0/1/2 and S
GM(D; T)P	Guanosine mono(di-; tri-)phosphate
His	Histidine
HU	Hydroxyurea
IF	Immunofluorescence
IP	Immunoprecipitation
IR	Ionizing radiation
IRES	Internal ribosomal entry site
KI	Knock-in
MCM	Minichromosome maintenance complex
Mec1	Mitosis entry checkpoint protein 1
MEF	Mouse embryonic fibroblasts
MS	Mass spectrometry

MYC	Myelocytomatosis oncogene
NAD	Nicotinamide adenine dinucleotide
NSP	Nucleotide salvage pathway
NTD	N-terminal domain
PCR	Polymerase chain reaction
PTEN	Phosphatase and tensin homolog
RF	Replication fork
RFX1	MHC class II regulatory factor 1
RNR	Ribonucleotide reductase
ROS	Reactive oxygen species
RPA	Replication protein A
RS(R)	Replication stress (response)
SCF complex	Skp, Cullin, F-box containing complex
Sml1	Suppressor of Mec1 lethality 1
ssDNA	Single stranded DNA
s-site	Specificity site
Strep	Streptavidine
siRNA	Small interfering RNA

TM(D; T)P	Thymidine mono(di-; tri-)phosphate
UV	Ultraviolet radiation
wt	Wild type

PRESENTACIÒN

La replicación del ADN es un proceso que ha de ser controlado con precisión. Su desregulación genera un tipo de daño en el ADN conocido como estrés replicativo (ER). El ER se define como la acumulación de ADN de cadena sencilla (ssDNA) desprotegido en las horquillas de replicación (RF) que se han bloqueado. Debido a su naturaleza recombinogénica, el ssDNA puede causar reordenamientos genómicos, algunos de los cuales ocurren frecuentemente en cáncer. Una de las razones por las que se acumula ssDNA es un aporte insuficiente de nucleótidos (dNTPs) que limita la progresión de las ADN-polimerasas. De este modo, se ha propuesto que los niveles reducidos de dNTPs son una fuente de inestabilidad genómica en cáncer. Con objeto de proteger sus genomas, las células necesitan detectar y limitar la cantidad de ssDNA. ATR es la principal quinasa que responde a ER en mamíferos, lo que resulta en la activación de una cascada de fosforilaciones que limita el avance en el ciclo celular al tiempo que mantiene la integridad de las horquillas de replicación. Si bien no se conoce el mecanismo exacto por el que la activación de ATR suprime ER, los resultados obtenidos en levadura sugieren que ATR podría estimular la producción de nucleótidos y así limitar el ER. En *Saccharomyces cerevisiae*, la mutación del ortólogo de ATR, Mec1, es letal y este fenotipo puede ser rescatado mediante la deleción simultánea de un inhibidor de la enzima ribonucleótido reductasa (RNR), Suppressor of Mec1 lethality 1 (Sml1). En todos los organismos eucariotas la RNR cataliza un paso limitante en la síntesis de dNTPs, reduciendo NDPs a dNDPs en un proceso que está altamente regulado. La RNR es un complejo heterotetramérico compuesto por una subunidad catalítica (Rnr1 o RRM1; en levadura o ratón, respectivamente) y una subunidad reguladora (Rnr2 o RRM2; en levadura o ratón, respectivamente), cuya estructura y función están muy conservadas a lo largo de la evolución. A pesar de que la secuencia que Sml1 reconoce en Rnr1 está conservada desde levaduras a mamíferos, y a pesar de que Sml1 de levadura une e inhibe a la RNR de mamíferos *in vitro*, se desconoce si la regulación de la RNR por parte de ATR se conserva más allá de levaduras.

En este trabajo hemos investigado la posible relación entre ER, ATR y la actividad RNR, y hemos buscado nuevos reguladores de la RNR en mamíferos. Si bien no hemos conseguido identificar nuevos reguladores de la RNR, hemos establecido que un incremento de la actividad RNR alivia los fenotipos ocasionados por una deficiencia en ATR *in vitro* e *in vivo*, lo que indica que la regulación de los reservorios de dNTPs por parte de ATR está conservada en mamíferos. Además, hemos mostrado que la mutación de un sólo residuo conservado de RRM1 evita la interacción de esta proteína con RRM2, y causa letalidad embrionaria temprana en ratones. A pesar del impacto de esta mutación, los ratones $Rrm1^{+/WG}$ no presentan un fenotipo obvio, lo que sugiere que RRM1 está presente en exceso en células de mamífero. Estos datos revelan que la interacción entre RRM1 y RRM2 es esencial para la viabilidad celular, y proveen el primer modelo genético de pérdida de función de la RNR en mamíferos.

ABSTRACT

DNA replication is a tightly controlled process with its misregulation leading to a type of DNA damage, known as replication stress (RS). RS is essentially defined as an accumulation of unprotected single-stranded DNA (ssDNA) at stalled replication forks (RF). Due to the recombinogenic nature of ssDNA, it is a cause of genomic rearrangements frequently observed in cancer. One way by which ssDNA can arise is an insufficient supply of nucleotides (dNTPs), which limits the progression of the DNA polymerases. Accordingly, reduced dNTP levels have been proposed as a source of genomic instability in cancer. In order to protect their genomes, cells need to detect and limit the amount of ssDNA. ATR is the initial kinase that responds to RS in mammals, resulting in a phosphorylation cascade that finally leads to cell cycle arrest and safeguarding of replication fork integrity. While it remains unclear how exactly ATR suppresses RS, evidences from yeast suggest that ATR might reduce RS through stimulating the production of nucleotides. In *Saccharomyces cerevisiae* (*S.cerevisiae*), lethality of the ATR ortholog Mec1 can be rescued by concomitant deletion of Suppressor of Mec1 lethality1 (Sml1), an inhibitor of the ribonucleotide reductase (RNR). In all eukaryotes, RNR catalyzes a rate limiting step in dNTP synthesis by reducing NDPs to dNDPs in a tightly controlled process. The RNR is a heterotetrameric complex composed of a catalytic subunit (Rnr1 and RRM1 in yeast and mouse respectively) and a regulatory subunit (Rnr2 and RRM2 in yeast and mosue respectively) whose structure and function are well conserved throughout evolution. Although the sequence where Sml1 binds to Rnr1 is conserved in mammals and yeast Sml1 binds and inhibits the mammalian RNR, it remains unclear whether ATR-dependent regulation of RNR is conserved beyond yeast.

In this work we investigated the putative relationship between RS, ATR and RNR activity, and searched for mammalian regulators of the RNR. Although we failed to identify new regulators of the RNR, we could establish that increased RNR activity

alleviates phenotypes associated with ATR-deficiency in mice *in vitro* and *in vivo*, pointing towards a functional conservation of the ATR-dependent regulation of the dNTP-pool in mammals. In addition, we show that a point mutation within a single conserved residue in RRM1 prevents binding to RRM2 causing early embryonic lethality in mice. Despite the impact of the mutation, $Rrm1^{+/WG}$ mice present no obvious phenotype suggesting that RRM1 exists in excess in mammalian cells. This data reveals that binding of RRM1 to RRM2 is essential for viability, and provides the first loss-of-function model of the RNR complex for genetic studies in mammals.

INTRODUCTION

1 Nucleotide pools and genome integrity

1.1 Replication Stress

DNA is the vehicle that stores all the genetic information of a cell and this information needs to remain intact over generations. While maintenance of genomic integrity is essential for cell viability, loss or change of this information in the form of mutations is the driving force behind evolution. Nevertheless, mutations present a threat to genomic integrity. DNA is constantly exposed to mutagenic environmental agents such as ionizing radiation (IR), ultra violet (UV) radiation and reactive oxygen species (ROS). Additionally, a large part of DNA damage is caused by endogenous sources such as cellular metabolites (De Bont and van Larebeke, 2004) or the DNA replication process itself. Precisely orchestrated and complete DNA replication during every cell cycle is crucial and its misregulation leads to a type of DNA damage known as replication stress (RS). RS is defined as an accumulation of unprotected single-stranded DNA (ssDNA) at stalled replication forks (RFs). Unprotected ssDNA is prone to anneal with another strand, even an incorrect one if the RF keeps stalled. Moreover, stalled RFs are resolved through recombination, which generates double-strand breaks (DSBs) that threaten genome integrity. As a consequence, high levels of RS lead to mutagenesis and genome instability, a known cause of many human diseases such as cancer and premature ageing (Lecona and Fernandez-Capetillo, 2014; Zeman and Cimprich, 2014). Fork progression can be hindered by multiple causes such as difficult to replicate regions or by an insufficient supply of nucleotides (dNTPs) (Byun et al., 2005; Friedel et al., 2009; Paulsen and Cimprich, 2007; Tourriere and Pasero, 2007). The result of any of those conditions is the stalling of the RF while the replicative helicase (MCM2-7 complex) continues to unwind DNA, leading to an accumulation of persistent ssDNA (Byun et al., 2005; Nedelcheva et al., 2005; Sogo

et al., 2002). Due to the threat ssDNA poses to the cellular genome integrity, mechanisms have evolved to protect from and resolve RS structures, the so called RS response (RSR) (Harrison and Haber, 2006; Zhou and Elledge, 2000). Activation of the RSR is mediated through a phosphorylation cascade that signals to stabilize and restore RFs, control DNA damage response genes, repair pathway choices and cell cycle progression (Reviewed in (Lopez-Contreras and Fernandez-Capetillo, 2010; Segurado and Tercero, 2009). The RSR is initially triggered by ssDNA, which becomes rapidly coated by the ssDNA-binding protein RPA. RPA-bound ssDNA brings ATRIP (ATR interacting protein), the constitutive binding partner of ATR, to the lesion (Cortez et al., 2001). In parallel, ssDNA-bound RPA also recruits the clamp loader RAD17 which in turn loads the Rad9-Rad1-Hus1 ring (9-1-1) onto the damaged site (Yang and Zou, 2006). Then, RAD17 recruits TopBP1 to activate ATR (Kumagai et al., 2006). In addition, RAD17 brings the adaptor protein Claspin to ssDNA, a step essential for the ATR-dependent phosphorylation of the effector kinase CHK1 (Wang et al., 2006). The DNA damage signal is extended to the rest of the nucleus via CHK1 signaling leading to stabilization and restart of the RF while arresting the cell cycle until the lesion is repaired (Cimprich and Cortez, 2008; Lopez-Contreras and Fernandez-Capetillo, 2010) **(Figure 1).** Of note, RS-free replication does not exist and as a consequence, ATR and CHK1 are essential for cell viability. In their absence, stalled forks that arise every S-phase cannot be stabilized and persist, causing a firing of backup origins which leads to a depletion of the dNTP pool, exacerbating RS and finally resulting in catastrophic fork collapse (Brown and Baltimore, 2000, 2003; Liu et al., 2000; Takai et al., 2000).

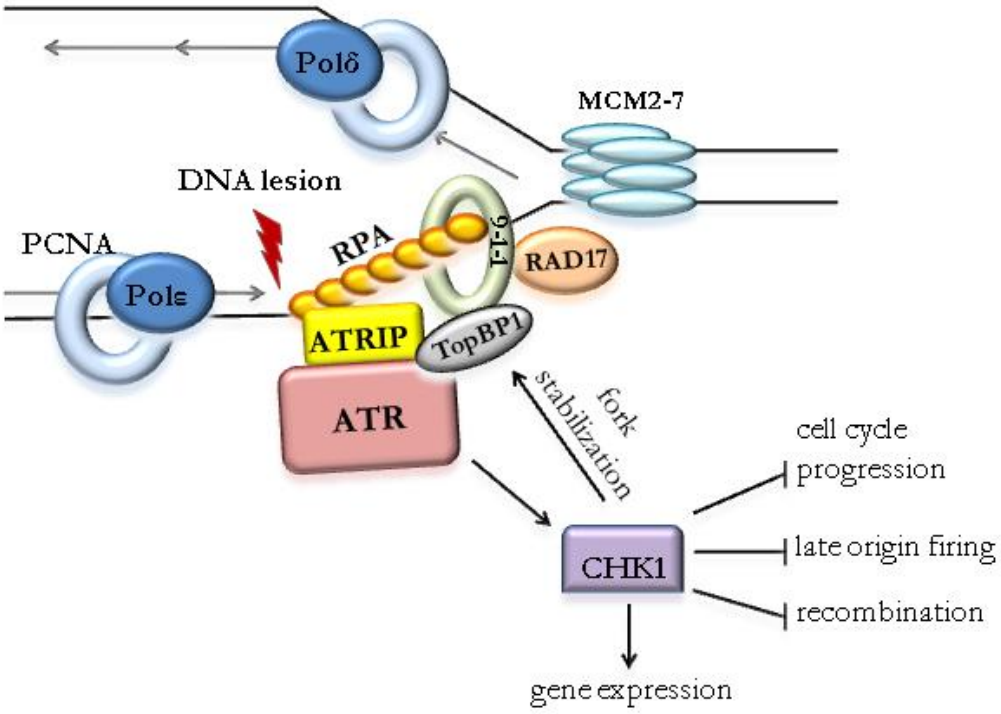


Figure 1: The ATR-mediated RS response maintains fork stability and orchestrates cell cycle progression. When progression of the RF is obstructed by a DNA lesions or a lack of dNTPs, the MCM2-7 complex may uncouple from the DNA-polymerases exposing long stretches of unprotected ssDNA (Byun et al 2005). This ssDNA is the activating signal for the RS response. RPA binds to the ssDNA, leading to binding of the ATR regulatory subunit ATRIP which in turn recruits ATR to the stalled fork. RPA is also the trigger for recruitment of Rad17 and a subsequent loading of the Rad-9 Hus-1 Rad-1 clamp (9-1-1). Together, they signal to recruit and position TopBP1, the allosteric activator of ATR. ATR phosphorylates and activates its downstream kinase CHK1, which extends the RS signal throughout the nucleus leading to fork stabilization, inhibition of cell cycle progression, late origin firing and homologous recombination as well as an induction of RS-response genes.

1.2 A role for the RSR in the regulation of the dNTP pool

Although ATR signaling has been an intense field of study, it remains mysterious how exactly it stabilizes stalled forks and limits RS. Proposed mechanisms include ATR-dependent regulation of the timely progression through the cell cycle (Liu et al., 2000), recombination-dependent restart of stalled RFs (Dehe et al., 2013) and regulation of replication factors (Lou et al., 2008). Data from yeast also suggest that ATR regulates dNTP pools to promote the coating of unprotected ssDNA (Zhao

et al., 2001; Zhao et al., 1998). In recent years, it has become increasingly clear that aberrant dNTP pools cause RS. In fact, some of the oldest anti-cancer drugs such as hydroxyurea (HU) and 5-fluorouracil induce RS though nucleotide pool depletion by targeting dNTP metabolic enzymes (Arlt et al., 2011; Gagou et al., 2010). Studies in yeast indicate that one of the essential roles of the ATR ortholog Mec1 is the induction of nucleotide production to reduce RS (Zhao et al., 2001; Zhao et al., 1998). In *S.cerevisiae*, the lethality of the deletion of the ATR ortholog Mec1 can be rescued either by supplementation with nucleotides or by mutations that increase the activity or the levels of the key enzyme in dNTP production, the ribonucleotide reductase (RNR). For instance, viability of *mec1Δ* is rescued upon concomitant depletion of Crt1, the transcriptional repressor of the RNR subunits, or upon depletion of its allosteric inhibitor Sml1 (Huang et al., 1998; Zhao et al., 2001; Zhao et al., 1998). Transcriptional regulation of RNR genes by ATR through the Mec1-Rad53-Dun1-RNR pathway is conserved to some extent in mammals through the ATR-CHK1-E2F1-RNR signaling **(see Introduction 2.2.2)**, while no mammalian ortholog of Sml1 has been identified to date. However, recent studies provide proof that addition of nucleosides can alleviate RS in vertebrates (Bester et al., 2011; Danilova et al., 2014). Nevertheless, to which degree ATR acts on the dNTP pool in mammals and whether this is one of its essential functions remains unclear.

1.3 Alterations in the nucleotide pools cause genomic instability

Optimal dNTP pools are critical for the fidelity of DNA replication and repair processes (Anglana et al., 2003) and their misregulation can lead to mutagenesis (Bester et al., 2011; Niida et al., 2010b). The control of dNTP pools is mainly achieved via allosteric and transcriptional regulation of enzymes involved in dNTP metabolism. Malfunctioning of those control mechanisms leads to altered dNTP pools resulting in enhanced mutagenesis, DNA recombination, chromosomal aberrations and cell death (Kunz et al., 1994). An excess of dNTPs primarily causes replication errors by driving misinsertions and promoting translesion DNA synthesis (Mathews, 2006), while dNTP scarcity slows down RF progression and

increases the frequency of fork stalling (Koc et al., 2004). dNTP levels also influence replication by affecting origin choice, inter-origin distance and dormant-origin usage (Anglana et al., 2003; Courbet et al., 2008; Ge et al., 2007). Many oncogenes, such as *Ras, Myc* and *cyclinE* induce RS by forcing DNA hyper replication and excessive origin firing that results in the accumulation of partly replicated DNA and increased DNA damage (Di Micco et al., 2006; Dominguez-Sola et al., 2007; Jones et al., 2013). Recently, it was proposed that this so called oncogene-induced RS arises as a consequence of insufficient dNTP levels caused by an unscheduled activation of DNA replication (Bester et al., 2011). Normal cells react to low nucleotide levels (e.g. induced by HU treatment) by activating the RS checkpoint and reducing fork speed resulting in a prolongation of S-phase (Alvino et al., 2007; Koc et al., 2004), a mechanism that preserves the nucleotide pools and protects genome integrity (Bianchi et al., 1986; Koc et al., 2004; Matsumoto et al., 1990). Many tumor cells have an inactive G1/S checkpoint and DNA synthesis is initiated in the presence of insufficient dNTPs causing excessive RS, increased DNA damage and accumulation of tumor promoting mutations (Sabatinos et al., 2012). While increased genomic instability due to mild levels of RS can enhance tumorigenesis, high levels of RS lead to unreplicated regions in the genome, hampering mitosis and resulting in mitotic catastrophe. Therefore, cancer cells are particularly dependent on a functional RS-response and increased levels of DNA precursors (Bester et al., 2011; Lecona and Fernandez-Capetillo, 2014). Accordingly, a mild increase in CHK1 levels protects cells from oncogene-induced RS and improves transformation efficacy (Lopez-Contreras et al., 2012). Similarly, changes in dNTP production through the alteration of key metabolic enzymes is a recurring feature in onco- and tumor-suppressor genes respectively (Aird and Zhang, 2015; Angus et al., 2002; Aye et al., 2015). High dNTP levels are mutagenic, but in contrast to low dNTP levels, compatible with survival. In yeast, upregulation of RNR activity seems to be a general response to RS (Davidson et al., 2012) and mutants with increased dNTP pools show improved survival following DNA damage while exhibiting higher mutation rates (Chabes et al., 2003a). In mammals, rate-limiting enzymes for dNTP production such as RNR and thymidylate synthase

(TS) are under the direct control of the *E2F* and *Myc* oncogenes and are commonly upregulated in human cancers (Aye et al., 2015). In addition, RNR activity has been proposed to play a role in overcoming oncogene-induced senescence in neoplastic cells (Aird and Zhang, 2015; Mannava et al., 2012; Mannava et al., 2013). The retinoblastoma tumor suppressor RB on the other hand, reduces expression of dehydrofolate reductase, RNR and TS (Angus et al., 2002). Hence, appropriate dNTP levels are required for normal cell proliferation and repair and excessive dNTP levels become essential after malignant transformation to sustain cancer progression.

2 Nucleotide biosynthesis

Nucleotides are composed of a nitrogenous base coupled to a five-carbon sugar (ribose or deoxyribose) (nucleoside) and one to three phosphate groups (nucleotide mono-, di-, tri-phosphate). Nucleotides have various functions essential for cell viability acting as an energy source (ATP, GTP and CTP), as part of coenzymes (AMP in NAD or Coenzyme A), as structural components of phospholipids or, and most prominently, as building blocks of DNA and RNA. In mammals, nucleotides can be newly synthesized through the *de novo* dNTP production pathway (DNP) or reused in the nucleoside salvage pathway (NSP).

2.1 De novo dNTP production

In the *de novo* pathway, both purines (Adenosine, Guanosine) and pyrimidines (Cytidine, Uridine, Thymidine) are synthesized from 5'-phospho α-D-ribosyl-1-pyrophosphate (PRPP) and amino acids (Mathews, 2006). Purine production involves the synthesis of inosine monophosphate (IMP) that is then converted via a separate two-step process into either AMP or GMP, which are subsequently phosphorylated to the corresponding NDP or NTP via specific kinases. Pyrimidine synthesis begins with the generation of the nitrogenous base orotate as free bases starting from orotic acid that is converted to its nucleotide OMP. OMP decarboxylation yields UMP that becomes subsequently phosphorylated to UTP.

Addition of glutamine to UTP generates CTP while dTMP is formed from dUMP **(Figure 2)**.

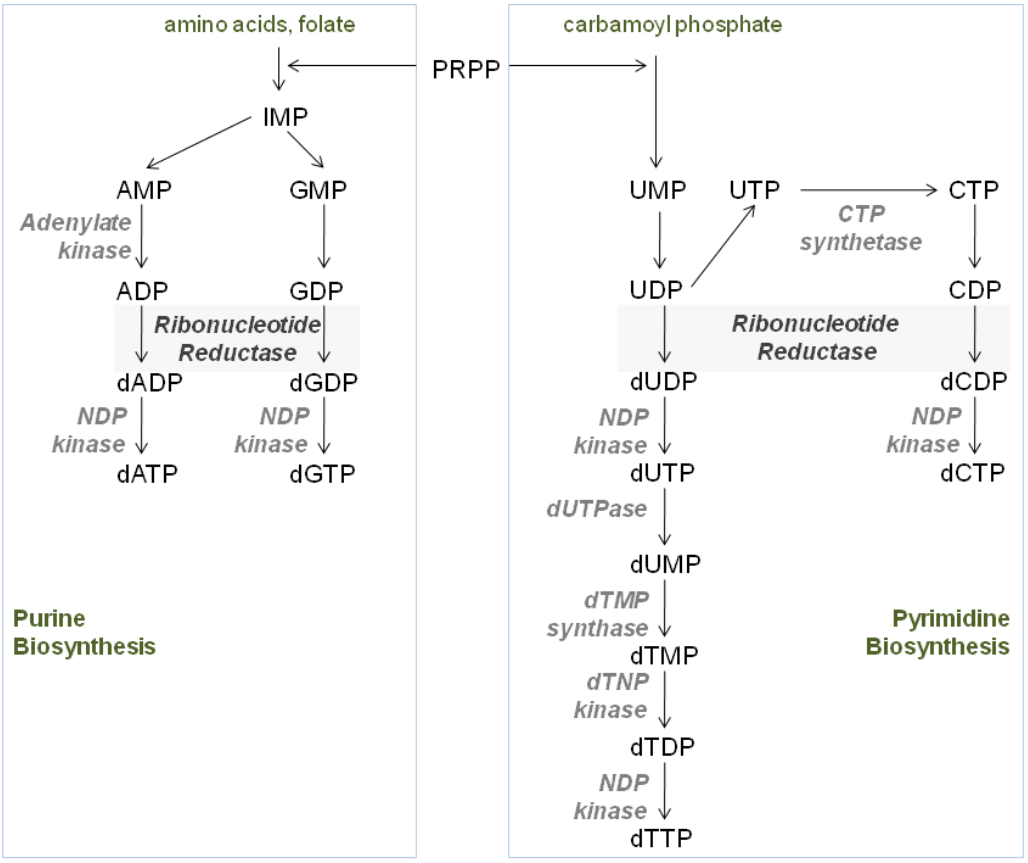


Figure 2: Purine and pyrimidine *de novo* dNTP synthesis in mammalian cells. Each enzyme name is in gray italics (Modified from (Mathews, 2006)).

2.2 Ribonucleotide reductase

RNR catalyzes the rate-limiting step in *de novo* dNTP synthesis **(Figure 2)** and the first step specifically dedicated to DNA synthesis by reducing all four types of NDPs to dNDPs **(Figure 3)** in a tightly controlled process. In fact, and although dNTP production is a multistep process including many different enzymes **(see Figure 2)**, the balance of the dNTP pool is primarily determined by the regulatory mechanisms of the RNR (Mathews, 2006).

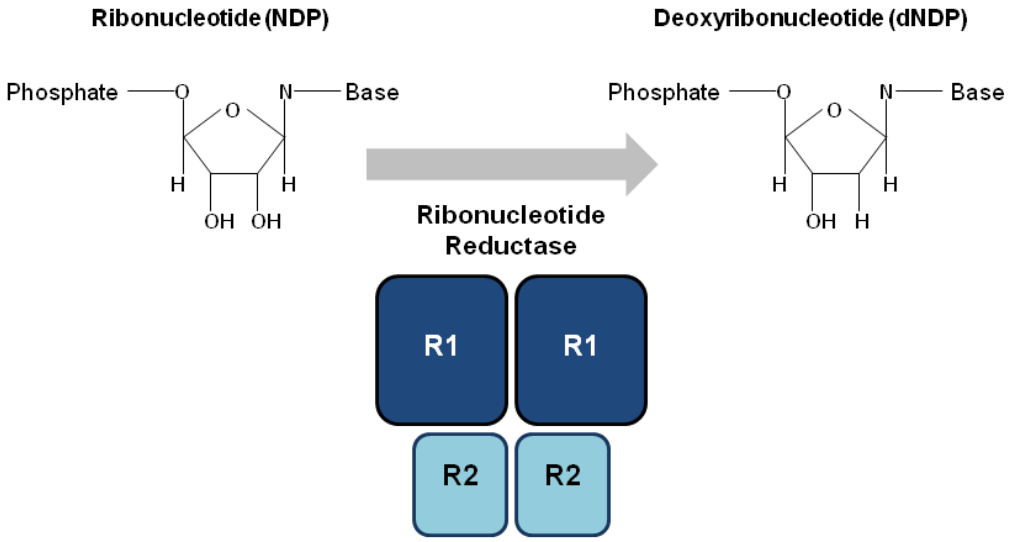


Figure 3: Ribonucleotide reductase (RNR) catalyzes the rate limiting step in *de novo* synthesis of dNTPs. Catalysis of ribonucleoside 5'-diphosphate (NDP) involves the reduction of the hydroxyl group at the 2'-carbon of ribose 5-phosphate to form the 2'-deoxy derivative-reduced 2'-deoxyribonucleoside 5'-diphosphate (dNDP).

There are three classes of RNR enzymes, distinguished by their catalytic mechanism. Eukaryotes (and some prokaryotes like *Escherichia coli* and viruses) contain Class-I RNRs, a heterotetrameric complex composed of two non-identical dimeric subunits. The minimal catalytically active RNR is an α2β2 complex (Fu et al., 2013; Minnihan et al., 2013; Uhlin and Eklund, 1994), although the α6β2 form has been proposed as the predominant form under physiologic conditions (Rofougaran et al., 2006). The α and β subunits are encoded by the R1 and R2 genes, respectively. The large subunit R1 (Rnr1 and RRM1 in yeast and mouse respectively) contains the catalytic site for reduction of the nucleotide as well as two allosteric sites for its regulation. The small subunit R2 (Rnr2 and RRM2 in yeast and mouse) carries a non-heme iron center (Fe-O-Fe) for the oxygen-dependent generation of a stable tyrosyl radical necessary for the reduction step. While each R2 monomer carries the di-iron center, only one tyrosyl radical is formed during each reduction cycle (Cotruvo and Stubbe, 2011). During catalysis, the radical is continuously shuttled via a long range proton-coupled electron transport chain to redox-active cysteines in the R1 active site to generate the thiyl

radical that reduces NDPs (Minnihan et al., 2013; Nordlund and Reichard, 2006). The active site, oxidized and inactive after this step, becomes subsequently activated by re-reduction of the cysteines via interaction with the R1 C-terminal domain (CTD) of the neighboring R1 subunit, preparing it for a new cycle of catalysis (Zhang et al., 2007).

2.2.1 Allosteric regulation of the RNR

The complex mechanism of allosteric regulation of the RNR was discovered long ago by Brown and Reichard (Brown and Reichard, 1969). The RNR contains two allosteric sites, both located in the R1 subunit. Overall enzyme activity is controlled by the activity site (a-site), while substrate choice is determined by the specificity site (s-site). The a-site is an ATP cone domain located in the N-terminus of the R1 subunit (Aravind et al., 2000). It is activated by ATP and inhibited by dATP binding, turning off nucleotide production whenever dNTP levels rise above a certain threshold (Nordlund and Reichard, 2006). The importance of the a-site specific regulation is exemplified by a mutation in the a-site that abolishes dATP feedback inhibition in yeast. This mutation leads to a 3-fold increase in the pyrimidine and a 9-fold increase in the purine dNTP pool accompanied by a 100 fold increased mutation rate (Weinberg et al., 1981). The s-site binds ATP, dATP, dTTP and dGTP; ATP and dATP binding favors the reduction of CDP and UDP, whereas dTTP and dGTP stimulate GDP and ADP reduction, respectively. The s-site is located at the R1-dimer interface and acts as a sensor to detect the concentration of each dNTP. Binding of each allosteric effector to the s-site induces a conformational change in a flexible loop, transmitting the specific signal to the catalytic site and thus making it more amenable for the corresponding substrate **(Figure 4)**. The interaction between the s-site and its allosteric effectors has been proposed to induce the dimerization of the R1 subunits and the subsequent RNR heterocomplex assembly (Rofougaran et al., 2006). The s-site specific regulation is important as the physiological equilibrium of dNTP levels may vary between organisms, but its

accuracy is crucial for genome integrity (Hofer et al., 2012; Nordlund and Reichard, 2006).

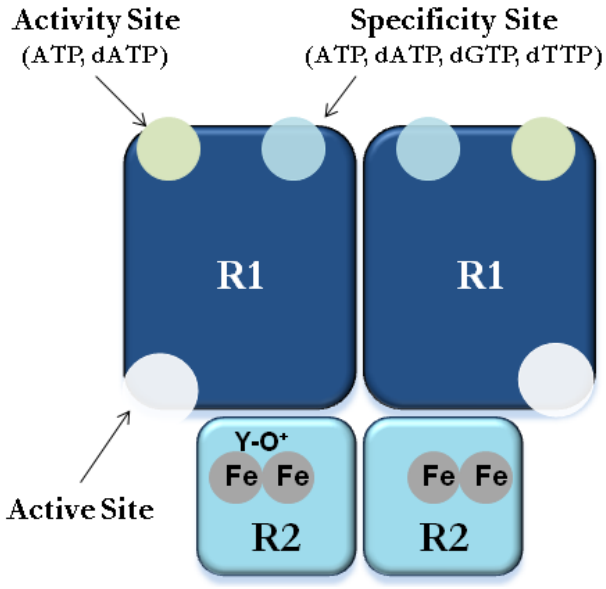


Figure 4: Schematic view of the RNR holoenzyme. The RNR heterotetramer is formed by two large R1 subunits and two small R2 subunits. R1 contains the active site for NDP reduction and two allosteric sites that regulate overall enzyme activity (activity site) and substrate specificity (specificity site). RNR is active when ATP is bound to the a-site and interaction of ATP, dATP, dGTP or dTTP with the s-site induce reduction of the according nucleotide. The R2 subunit contains a di-ferric iron center (Fe-Fe) and one subunit forms the protein tyrosyl radical(Y-O•) that is transferred to the R1 subunit for catalysis.

2.2.2 RNR transcriptional and post-transcriptional regulation

Aside from its sophisticated allosteric control mechanism, multiple levels of RNR regulation exist. They function in concert to strictly limit RNR activity to those stages of the cell cycle when the requirement for dNTPs is highest, namely S/G2-phase and during DNA damage repair. As a consequence, RNR protein levels and activity start to rise at the G1/S border, resulting in a ten-fold increase in dNTP levels, which decrease before cells enter into mitosis (Mathews, 2006). The cell cycle restricted activity of RNR is observed from yeast to human and achieved via conserved as well as distinct regulatory mechanisms, including: transcription,

protein inhibitor interaction, protein degradation and control of subcellular localization.

RNR transcriptional regulation in yeast

In budding yeast (*S.cerevisiae*), the R1 dimer is formed by the products of two genes, *rnr1* and *rnr3*. Only *rnr1* is essential for viability forming the Rnr1 homodimer under standard conditions, while Rnr3 is normally present at low levels. In response to DNA damage or replication block, Mec1 is activated and phosphorylates Rad53. Rad53, in turn, phosphorylates the Dun1 kinase that acts on Crt1, a transcriptional repressor of different rnr genes as well as of its own promoter. Crt1 phosphorylation induces its release from DNA, leading to transcriptional activation of its target genes (Lubelsky et al., 2005). As a consequence, *rnr3* is induced and accumulates in the cytoplasm, allowing the formation of an alternative R1 dimer (Mathews, 2006; Wu and Huang, 2008). In contrast, transcription of *rnr1* is regulated by the dimeric MluI-binding factor (MBF) composed of the regulatory transactivating protein Swi6 and its DNA binding partner Mbp1 (Reviewed in (de Bruin and Wittenberg, 2009)). In addition, *rnr1* expression is controlled during the cell cycle and in response to DNA damage by the high-mobility group transcription factor Ixr1 (Tsaponina et al., 2011). In budding yeast, the R2 subunit is a constitutive heterodimer formed by the products of two essential genes, *rnr2* and *rnr4* (Huang and Elledge, 1997; Perlstein et al., 2005; Sommerhalter et al., 2004). Only Rnr2 is able to form the diferric tyrosyl cofactor while Rnr4 lost this ability but is essential for radical generation and stabilization of the heterodimer (Chabes et al., 2000; Perlstein et al., 2005; Voegtli et al., 2001). Like *rnr3*, *rnr2* and *rnr4* are under the control of the Crt1 repressor and induced in response to DNA damage response (DDR) signaling (Huang et al., 1998; Zhao and Rothstein, 2002) **(Figure 5).**

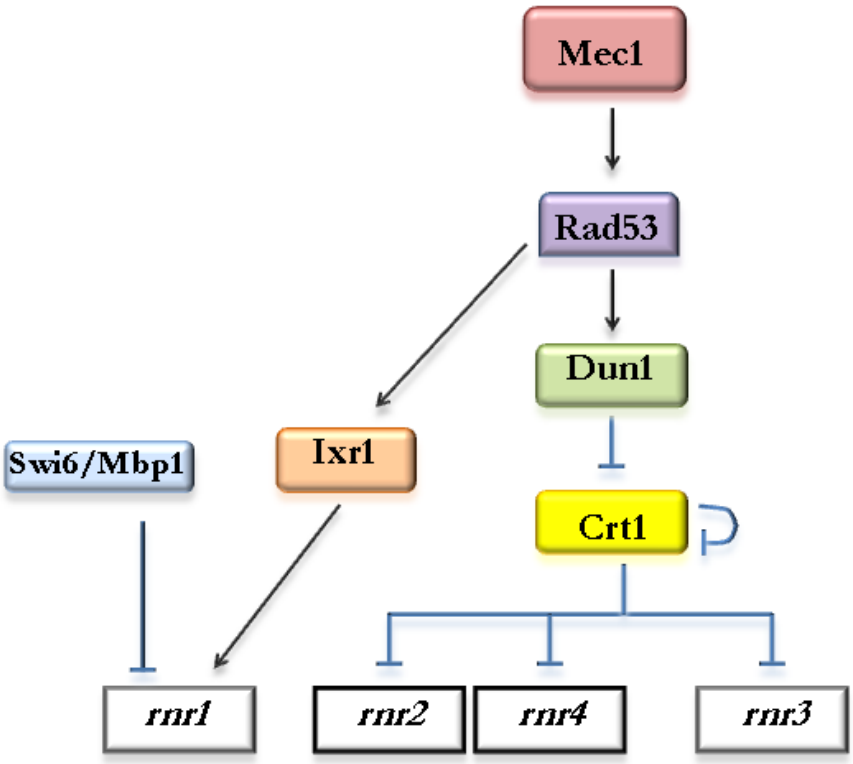


Figure 5: Transcriptional regulation of RNR in *S.cerevisiae*. Mec1 signaling induces a phosphorylation cascade that activates the downstream kinases Rad53 and Dun1 which finally leads to a transcriptional de-repression of the rnr genes *rnr2-4*. Expression of *rnr1* depends on Mec1 and Rad53 but not on Dun1 and is instead regulated through Ixr1 and the Mec1-independent MBF heterocomplex (Modified from (Sanvisens et al., 2013)).

RNR post-transcriptional regulation in yeast

In budding yeast, an additional level of RNR regulation is achieved through the interaction with a small allosteric inhibitor, the Sml1 protein (Chabes et al., 1999; Zhao et al., 1998). Sml1 specifically binds to the large R1 subunit, inhibiting dNTP production whenever DNA synthesis is not desired (Chabes et al., 1999). Upon entry into S-phase or in response to DNA damage, Mec1 signaling activates the downstream kinase Dun1, which in turn phosphorylates Sml1. Phosphorylated Sml1 becomes ubiquitinated by the E2/E3 ubiquitin ligase complex Rad6-Hbr2-Hub1, resulting in its proteasomal degradation. Zhang and colleagues propose a model where Sml1 competes with the R1-CTD for the interaction with the N-

terminal domain (NTD) of the neighboring R1, blocking an essential step for complex regeneration (Zhang et al., 2007) **(Figure 6)**.

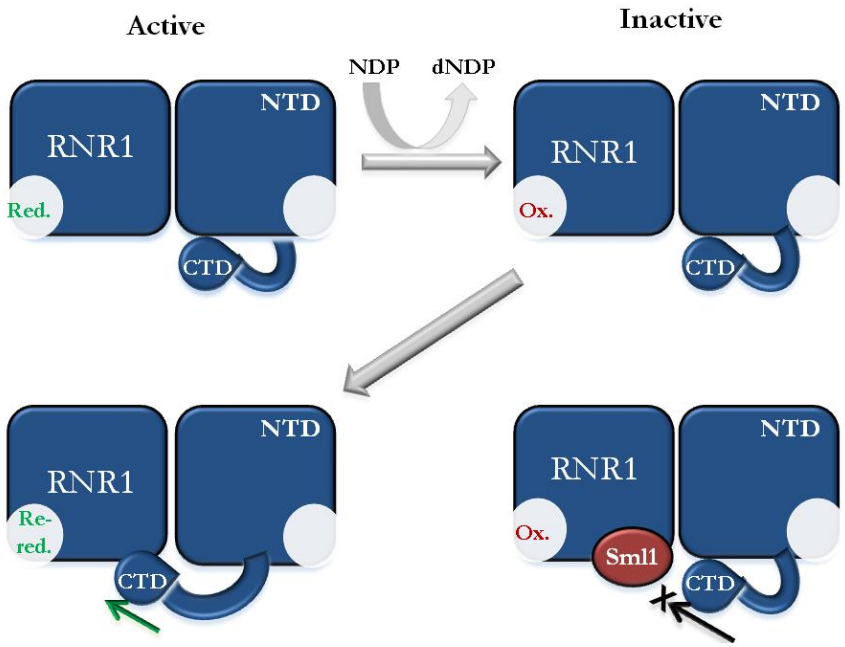


Figure 6: A model for the inhibition of Rnr1 by Sml1. The active site becomes oxidized and inactive after each catalytic cycle. Interaction of the C-terminal domain (CTD) of the neighboring R1 with a conserved region in the R1-N-terminal domain (NTD) is essential for re-reduction and re-activation of the RNR. Sml1 competes against R1-CTD for the binding to this region in the R1-NTD, blocking regeneration of the complex (Modified from (Zhang et al., 2007).

Under unperturbed conditions, Sml1 is present as a free homodimer and in equilibrium with the inactive Sml1-Rnr1 complex. Upon Mec1 initiated degradation of free Sml1, the equilibrium shifts towards an active RNR that can now produce dNTPs for DNA replication or repair (Zhao et al., 2001; Zhao and Rothstein, 2002). The removal of the RNR inhibitor provides a much faster mechanism to induce dNTP production compared to transcriptional regulation and its significance is underlined by the fact that deletion of Sml1 rescues the lethality of *mec1Δ* strains (Zhao et al., 1998).

RNR localization in yeast

In both fission and budding yeast, the R2 subunit localizes predominantly to the nucleus outside of S-phase and relocalizes to the cytoplasm in response to S-phase entry or DNA damage checkpoint activation (Liu et al., 2003). Since R1 constitutively localizes to the cytoplasm, R2 relocalization is a prerequisite for RNR complex formation and dNTP production (Nestoras et al., 2010). In *S.cerevisiae*, Dif1 (Damage regulated import factor 1) regulates Rnr2-nuclear import (Lee et al., 2008; Wu and Huang, 2008) while nuclear sequestration of Rnr2 is achieved through binding to the WD40 repeat protein Wtm1 (Lee and Elledge, 2006). In response to S-phase entry or DNA damage, Dif1 becomes phosphorylated and degraded in parallel. The Wtm1-Rnr2 interaction is weakened while nuclear export remains active leading to a cytoplasmic accumulation of Rnr2 with subsequent RNR complex formation and activation (Lee et al., 2008; Wu and Huang, 2008) **(Figure 7).** Dif1 and Sml1 are related genes and share a common domain, the Sml1 domain, involved in phosphorylation-dependent degradation (Lee et al., 2008; Wu and Huang, 2008). In addition, the *S.cerevisiae* RNR regulatory gene cluster contains a third gene, *Hug1*. Hug1 shares with Dif1 a sequence motif that is involved in binding to R2 (Lee et al., 2008; Nestoras et al., 2010) and that was suggested to fine-tune RNR activity (Meurisse et al., 2014). In *S.pombe*, Spd1 regulates both R2 nuclear import as well as inhibition of R1 (Hakansson et al., 2006a). Spd1 shows limited sequence homology to Dif1/Sml1/Hug1 in different functional regions (Lee et al., 2008): the proposed R1 interaction domain shows similarity to the Rnr1 binding domain of Sml1 (Nestoras et al., 2010; Zhao et al., 2000) and the R2-interaction domain presents homology to the corresponding region in Dif1/Hug1 (Lee et al., 2008; Nestoras et al., 2010). It has been proposed that gene duplication events of the ancestral RNR-regulatory locus led to a separation into three distinct genes in *S.cerevisiae* (Meurisse et al., 2014). The coordinated regulation of RNR activity allows a two-fold increase in the dNTP pool during replication or a 6-8 fold increase after the activation of the DDR, ensuring

efficient DNA replication and repair while preventing large dNTP pool expansions that might induce genotoxicity (Chabes and Thelander, 2003).

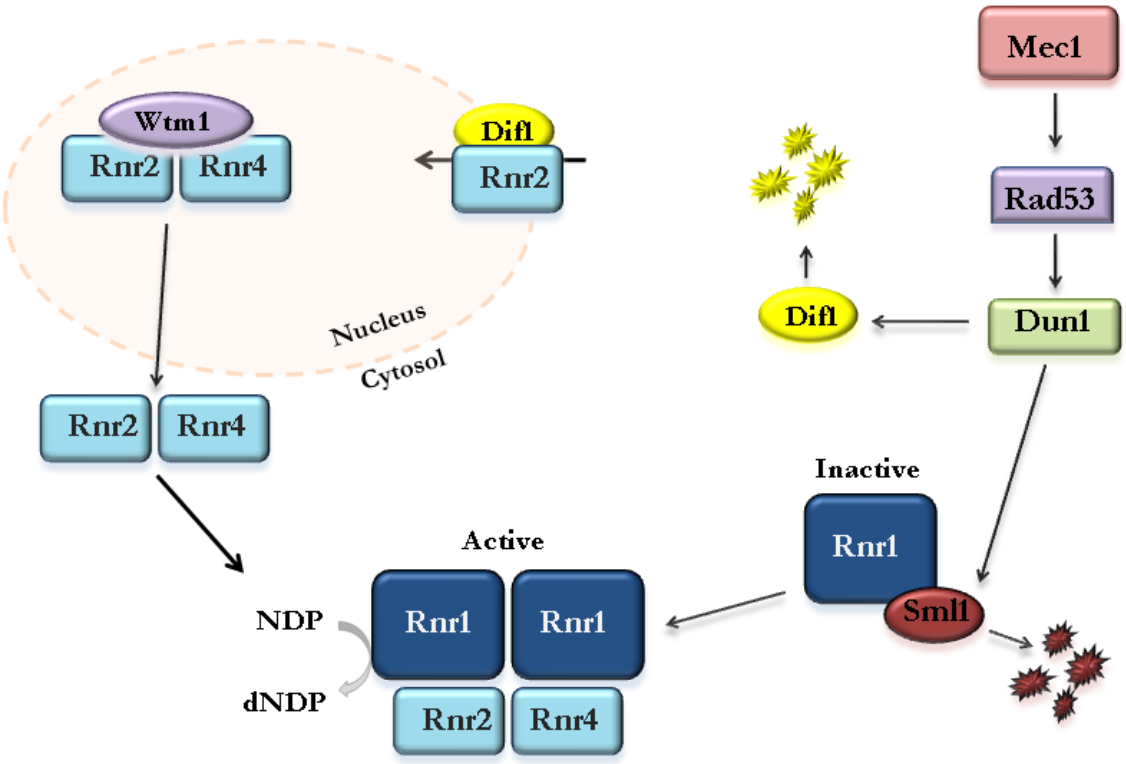


Figure 7: Post-transcriptional regulation of the RNR in *S.cerevisiae*. During S-phase and in response to genotoxic stress, Mec1 signaling phosphorylates and activates its downstream kinases Rad53 and Dun1 which leads to activation of RNR by (1) promoting the phosphorylation and degradation of the Rnr1-inhibitor Sml1 and (2) inducing Rnr2 cytosolic accumulation by degradation of its nuclear importer Dif1 and weakening the interaction of Rnr2 with Wtm1.

RNR transcriptional regulation in mammals

The mammalian RNR complex is a heterotetramer formed by two identical RRM1 subunits and two RRM2 subunits. Mammals contain an additional R2 gene, *p53R2*, which codes for a protein called RRM2B. *p53R2* was discovered in 2000 by two groups (Nakano et al., 2000; Tanaka et al., 2000) and shares about 80%-90% sequence homology and domain conservation with RRM2. RRM2B can form a functional complex with RRM1 and provides dNTPs for mitochondrial replication and DNA repair outside of S-phase (Lozano and Elledge, 2000; Nakano et al., 2000; Tanaka et al., 2000). The discovery of *p53R2* directly connected DDR signaling to

RNR activity in higher eukaryotes since *p53R2* is induced in response to p53 signaling and ATM phosphorylates and stabilizes RRM2B (Chang et al., 2008).

Transcription of *Rrm1* and *Rrm2* is cell cycle-dependent, with undetectable mRNA levels in G0/G1 phase and levels peaking in S-phase (Bjorklund et al., 1990). The Rrm1 promoter presents an Inr element and 3 control regions (β, α, and γ). Promoter strength is sustained by binding of the YY1 transcription factor to β and α regions while cell cycle-dependent regulation contributes to enhance promoter activity (Johansson et al., 1998). *Rrm2* contains a TATA-box within its core promoter region, an upstream promoter activating region crucial for overall activity, a proximal promoter binding element containing an E2F4 binding site and an adjacent NF-Y binding CCAAT motif (Filatov and Thelander, 1995). During G1, E2F4 binds to the conserved E2FII binding site, leading to repression of transcription. Upon entry into S-phase, E2F4 dissociates and relieves *Rrm2* repression leading to transcriptional activation (Chabes et al., 2004). Analog to the Mec1-Rad53-Dun1-dependent regulation of RNR genes in yeast, mammalian RNR genes seem to be under the control of ATR/ATM-CHK1-E2F signaling (Eaton et al., 2007; Zhang et al., 2009). Several studies report either no induction of *Rrm2* expression after DNA damage (Chabes and Thelander, 2000) or even inhibition by p53 (Lin et al., 2004; Tanaka et al., 2000; Yamaguchi et al., 2001). In contrast, others claim that *Rrm2* is induced in response to some DNA insults (UV light) but not to others (HU) (Filatov et al., 1996). However, the induction of *Rrm1* and *p53R2* expression in response to DNA damage is well established (Eaton et al., 2007; Nakano et al., 2000; Tanaka et al., 2000), and an increasing number of studies also report that *Rrm2* is indeed upregulated in response to DDR signaling. In this sense, the Crt1 mediated repression of RNR genes is functionally conserved in mammals. The mammalian ortholog of Crt1, Rfx1, binds and represses the *Rrm2* gene as well as its own promoter in unperturbed conditions, but is released upon CHK1 signaling resulting in de-repression of its target genes (Lubelsky et al., 2005). In contrast to *Rrm2*, the p53R2 gene is not cell cycle regulated but expressed at low levels throughout the cell cycle and highly induced in response to p53 signaling

(Nakano et al., 2000; Tanaka et al., 2000). In the absence of p53, *p53R2* is not induced. Instead, RRM2 levels increase to provide dNTPs for repair (Lin et al., 2004). Of note, efficient induction of *p53R2* expression takes roughly 24 hours leading to a less than 2-fold increase in the dNTP pool in G0/G1 (Hakansson et al., 2006b). DNA damage repair is thought to take place within hours after damage (Smith and Seo, 2002) indicating that other factors such as RNR localization or activation must provide sufficient dNTPs for DNA replication and repair.

RNR post-transcriptional regulation in mammals

Compared to yeast, mammals exhibit a more stringent allosteric regulation of the activity of RNR, and can further regulate dNTP pools via the salvage pathway **(see Introduction 2.3)** and a dNTP secretion system, pathways absent in yeast. Much less is known about post-transcriptional regulatory mechanisms of RNR activity in mammals and some of them remain controversial. Nevertheless, it is clear that mammalian cells also regulate the RNR at the post-transcriptional level. In contrast to yeast, overall activity of mammalian RNR is controlled through the modulation of the levels of the small subunit RRM2. Although expression of both, *Rrm1* and *Rrm2* is regulated in a cell cycle-dependent manner, the regulation of their protein levels differs greatly. RRM1 protein has a very long half life (>20 hours) resulting in almost constant levels that are thought to be in excess throughout the cell cycle (Engstrom et al., 1985). In contrast, RRM2 has a short half life (3 hours) and is only stable during S-phase and in response to DNA damage signaling. During G0/G1 and in late mitosis, RRM2 becomes ubiquitinated and degraded by the anaphase promoting complex (APC) (Chabes and Thelander, 2000; Chabes et al., 2003b). RRM2B lacks the KEN box, the sequence recognition motif for the APC present in the RRM2 sequence, and hence is not degraded. Recently, a study by D'Angiolella (D'Angiolella et al., 2012) reported an additional mechanism of RRM2 protein regulation via $SCF^{CyclinF}$-dependent degradation during G2. This study linked ATR signaling to RNR activity showing that ATR signaling decreases CyclinF levels, leading to a stabilization of RRM2 in G2 (D'Angiolella et al., 2012).

Regarding the allosteric inhibition of the mammalian RNR, two recent studies reported the interaction and inhibition of mammalian RNR by different proteins. Xie and colleagues found that the anti-apoptotic protein Bcl2 binds RRM2 and inhibits RNR complex formation causing reduced RNR activity, dNTP levels and RS (Xie et al., 2014). In a different study, a protein involved in ion-transport, IRBIT, was found to bind and inhibit the RNR, but this interaction was limited to the RRM1-RRM2B complex and could only be observed during mitosis (Arnaoutov and Dasso, 2014). While these studies propose the existence of new inhibitors of the mammalian RNR, their impact on RNR function during replication and whether and how they are linked to ATR signaling remains unknown.

RNR localization in mammals

Another unresolved question is whether RNR activity is controlled via subcellular localization of the complex in mammals (Pontarin et al., 2008). The bulk of RRM1 and RRM2 protein localizes to the cytoplasm. Hence, it is generally believed that nucleotide synthesis takes place in the cytosol and newly synthesized dNTPs enter the nucleus by diffusion (Engstrom and Rozell, 1988; Pontarin et al., 2008). However, several works have suggested that nucleotides could be synthesized in close proximity to RFs. For instance, Reddy and Pardee proposed nuclear translocation of the RNR (Prem veer Reddy and Pardee, 1980). In addition, Mathews and others found that the cellular concentration of dNTPs is about four times lower than that required for DNA synthesis (Mathews and Sinha, 1982; Warner, 1973) and speculated that the RNR might form part of the so called replisome. In this model the RNR and other dNTP synthesizing enzymes are associated to the replication machinery to provide a locally increased supply of DNA precursors at the site of synthesis. Studies in *E.coli* corroborate this hypothesis, as the R1 subunit co-localizes with replication factors (Sanchez-Romero et al., 2010). It is becoming increasingly clear that at least small fractions of the RNR subunits translocate to the nucleus to promote DNA damage repair. CHK1 signaling not only regulates RRM2 transcription but also induces RRM2 nuclear translocation (Zhang et al., 2009) and RRM2 and RRM2B were found to

enter the nucleus in human tumor cell lines (Liu et al., 2005). In 2010, Niida and colleagues showed that a small fraction of RRM1 and RRM2 associates with chromatin in a Tip60-dependent manner and are localized to sites of DSB (Niida et al., 2010a). Recently, Hu and colleagues demonstrated that the association of the thymidylate kinase (TMPK) and RNR to DNA lesions is required for DSB repair (Hu et al., 2012). Finally, D'Angiolella et al provided evidence of a nuclear accumulation of RRM2 dependent on ATR-induced stabilization of RRM2 during G2 (D'Angiolella et al., 2012). While the nuclear translocation of the RNR in response to DNA repair is now well established, it remains unclear whether the RNR also localizes to sites of DNA replication and RS.

2.3 The nucleotide salvage pathway

Apart from the DNP for dNTP synthesis, mammalian cells feature an additional RNR-independent pathway to generate nucleotides, the nucleotide salvage pathway (NSP). While actively replicating cells mainly rely on the DNP for nucleotide production, the NSP is the main source of dNTPs in quiescent cells (Kufe et al., 1984; Sandoval et al., 1996). The NSP recycles extracellular deoxyribonucleosides from nucleic acid degradation (DNA turnover and food intake) and apoptotic cells (Arner and Eriksson, 1995). The precursors are imported via specialized transporters and phosphorylation by specific kinases generates the final nucleotide (Reichard, 1988). Deoxycitidine kinase (dCK) phosphorylates deoxycitidine and, to a lesser extent, deoxyadenosine and deoxyguanosine, while thymidine kinase 1 (TK1) phosphorylates thymidine (Reichard, 1988). The NSP was long considered irrelevant for the regulation of dNTP pools (Xu et al., 1995) but, more recently, studies indicate that it might affect DNA replication and repair under certain conditions. Depletion of dCTP pools in $dCK^{-/-}$ mice leads to RS, S-phase arrest and DNA damage in hematopoietic progenitor cells, which can be rescued by concomitant depletion of TK1 (Austin et al., 2012; Toy et al., 2010). Although the bulk of NSP-derived free dNTPs does not contribute to DNA synthesis under unchallenged conditions (Nathanson et al.,

2014), dNTP synthesis via the NSP becomes critical for the maintenance of nucleotide levels when *de novo* dNTPs synthesis is inhibited (Austin et al., 2012). Moreover, dCK is a target of ATM and ATR kinases (Matsuoka et al., 2007), supporting that induction of the NSP pathway might be important to produce sufficient dNTPs in response to DNA damage and RS. The induction of an alternative dNTP production mechanism might also explain resistance towards the cytotoxic effects of drugs targeting dNTP *de novo* synthesis such as thymidine, which inhibits RNR-dependent pyrimidine synthesis (Reichard, 1988). Interestingly, a recent study showed that the combination of thymidine and dCK inhibitors can be efficient in treatment of hematological malignancies in mice (Nathanson et al., 2014). Therefore, concomitant targeting of DNP and NSP could provide an effective alternative to target cancer cells and reduce the development of resistances to treatment.

3 RNR in cancer and disease

As the key regulator of dNTP *de novo* biosynthesis, RNR plays a pivotal role in maintaining genome integrity. Altered regulation of its activity is highly mutagenic (Chabes et al., 2003a; Mathews, 2006) and its upregulation has been described in certain types of human cancer (Elford et al., 1970). However, the RNR subunits seem to play distinct roles in tumor development. RRM2, being considered the rate-limiting subunit of RNR, is overexpressed in a number of human cancers including gastric, ovarian, bladder and colorectal cancer (Lu et al., 2012; Morikawa et al., 2010; Wang et al., 2012). A clear oncogenic activity for RRM2 was described in human carcinoma cells *in vitro*, where high RRM2 expression enhanced invasive potential (Duxbury et al., 2004b) and in mouse, where increased focus formation and anchorage independent growth was enhanced by RRM2 overexpression (Fan et al., 1998; Fan et al., 1996).

Similarly, suppression of the alternative small subunit RRM2B inhibits cancer cell proliferation (Matsushita et al., 2012) and increased expression of *p53R2* is detected in melanoma, oral carcinoma, esophageal squamous cell carcinoma and

non-small cell lung cancer (NSCLC) (Matsushita et al., 2012; Okumura et al., 2006; Uramoto et al., 2006; Yanamoto et al., 2009; Yanamoto et al., 2003). In other cancers, such as colon adenocarcinoma and colorectal cancer, *p53R2* expression is negatively correlated with metastasis formation (Liu et al., 2006), cell invasiveness and survival (Liu et al., 2011).

In contrast to the small subunits, a tumor suppressive function has been proposed for the large RRM1 subunit and its overexpression reduces transformation *in vitro* (Fan et al., 1997). The RRM1 locus shows frequent loss of heterozygosity in lung cancers and high levels of RRM1 expression are associated with increased survival rates. Mouse models overexpressing RRM1 confirmed the tumor suppressor activity of RRM1 which seems to be mediated by improved DNA damage repair (Gautam and Bepler, 2006). However, it has been speculated that the tumor suppressive function might not be solely related to RRM1's function in dNTP production, as migration and invasion, tumorigenicity and metastasis are mediated via RRM1-dependent induction of PTEN (Gautam et al., 2003).

3.1 Targeting dNTP metabolism for cancer treatment

Owing to the increased proliferation rates and genomic instability of transformed cells, tumor cells are more sensitive to the cytotoxic effects of dNTP deprivation. Thus, RNR and other dNTP metabolic enzymes are major targets in several types of cancer treatments (Aye et al., 2015; Bonate et al., 2006; Zhu et al., 2009). In fact, the first chemotherapy ever developed was an antifolate, a compound which essentially limits dNTP production. Folates are B9 vitamins that serve as co-factors in multiple crucial biosynthetic pathways including *de novo* biosynthesis of purines and thymidylate and, as such, are essential for cell division, DNA synthesis and repair. Most antifolates inhibit the dihydrofolate reductase (DHFR), antagonizing the function of folic acid. In the 1940s, Sidney Farber discovered that antifolates can inhibit the proliferation of malignant cells and showed some effectiveness in the treatment of acute lymphoblastic leukemia (Farber and Diamond, 1948). Nowadays, a large number of newly designed antifolates are still in clinical use (e.g.

Methotrexate) (Walling, 2006) and various other antimetabolites such as nucleoside analogs have been developed for the treatment of cancer and other diseases.

Many nucleoside analogs inhibit the RNR subunit RRM1 by blocking either its active or allosteric sites (Reviewed in (Shao et al., 2013)). For instance, Gemcitabine (dFdC), a cytidine analog, is used in the clinic for the therapy against pancreatic, bladder and lung cancer (Reviewed in (Stubbe and van der Donk, 1995). As most nucleoside analogs, it is administered as a prodrug that needs to be phosphorylated in order to be active (Bonate et al., 2006). In the case of Gemcitabine, the diphosphate analog binds and inhibits the RRM1 active site irreversibly while the triphosphate can be incorporated into DNA leading to termination of chain elongation. The reduced dNTP levels caused by RRM1 inhibition lead to increased incorporation of the dNTP analog into the DNA potentiating its effect (Artin et al., 2009; Plunkett et al., 1995). Despite the success of nucleoside analogs in the clinic, they also feature some disadvantages (Alvarez-Salas, 2008). Overexpression of the target enzyme can result in resistance to the drug (Goan et al., 1999) and metabolism or catabolism of the prodrug and nucleos(t)ide analogs respectively can lead to dangerous side effects and toxicity (Gonzalez et al., 1998; King et al., 2006; Lindemalm et al., 2004). Because inhibition of the RNR can induce sensitivity to DNA damage-inducing agents, nucleoside analogs such as Gemcitabine are commonly used in combination therapy. For instance, in Carboplatin-resistant tumors a combination of Gemcitabine with Carboplatin (Pfisterer et al., 2005) has proven efficient to overcome resistances (Sandler and Ettinger, 1999).

In addition, RNR can be inhibited via targeting of the RRM2 subunit. Most RRM2 inhibitors are metal chelators and/or radical quencher affecting the tyrosyl radical or the iron center. HU inactivates RNR by targeting both, the tyrosyl radical and the iron center. It was first synthesized in 1869 and primarily used for myeloproliferative disorders which can develop into acute myeloid leukemia and for which it is still used in the clinic (Sterkers et al., 1998). HU is also used for the

treatment of chronic myelogenous leukemia (Hehlmann et al., 2011), glioblastoma (Levin, 1992) and in combination with radiotherapy in cervical and head and neck cancer. However, cancer cells frequently develop resistance by overexpressing RRM2 (Akerblom et al., 1981; McClarty et al., 1987; Wright et al., 1987). In addition, HU has a short half-life, low affinity for RNR and may target other metalloenzymes (Temperini et al., 2006). The iron chelator Triapine is a more potent inhibitor of RNR than HU and has shown promising results in clinical trials for combination treatment with cisplatin/radiation in human cervical and vaginal cancer (Kunos et al., 2010; Kunos et al., 2013). Triapine has also shown to be effective against HU-resistant tumors (Finch et al., 2000). Besides small molecule inhibitors, new targeting strategies are currently being investigated including RNR gene silencing approaches. Although this strategy is still limited by technical obstacles, such as inefficient siRNA-delivery in patients (Oh and Park, 2009), early clinical trials show promising results (Cao et al., 2003; Duxbury et al., 2004a; Rahman et al., 2012). Further improvements of the techniques, new combination therapiess and deeper understanding of the regulatory pathways controlling dNTP levels are likely to help improve cancer therapy in the future.

In summary, the adequate regulation of nucleotide levels by RNR is necessary for genomic integrity and its misregulation has emerged as an important source for RS in tumor development. How the RS checkpoint (ATR and CHK1) suppresses RS and whether this is at any level linked to dNTP biosynthesis is not known. However, evidences from yeast suggest that the essential function of the ATR ortholog Mec1 is indeed linked to the regulation of the dNTP pool. Whereas the structure and function of RNR are highly conserved during evolution, it remains unclear to what extent the ATR-dependent regulation of the RNR is conserved in mammals. Based on the above, we hypothesized that the essential roles of mammalian ATR might also relate to dNTP production. Verifying this putative relationship between ATR and RNR, and the search for mammalian regulators of RNR activity constitute the main objectives of my PhD.

OBJECTIVES

1. To investigate the contribution of ATR in regulating RNR activity in mammals.
    a. To determine the impact of ATR on the expression of RNR subunits.
    b. To explore whether nucleoside supplementation reduces the toxicity associated to limited ATR activity.
    c. To investigate whether increased RNR activity can rescue the phenotypes of ATR-Seckel mice.

2. To identify new regulators of the mammalian RNR.
    a. To perform proteomic analyses of purified mammalian RNR complexes.
    b. To explore if and how the putative interactors of the mammalian RNR identified in 2.a suppress replication stress in mammals.

3. To investigate the impact of the RRM1-W684G mutation in mice, which in yeast stabilizes the binding of the Rnr to its allosteric inhibitor Sml1.
    a. To generate mice carrying a W684G mutation in *Rrm1*.
    b. To analyze the impact of *Rrm1*-W684 heterozygosity or homozygosity in mice.
    c. To investigate the mechanisms by which RRM1-W684G expression limits the activity of the RNR in mammalian cells.

MATERIALS AND METHODS

1 Mouse work

1.1 Generation of the Rrm1^{WG} mouse model

For the generation of *RRM1^{KI}* mice a 16.8 kb region from the mouse genome which targeted the *RRM1* gene was first cloned from a BAC (BAC RP23-111K8) into a minimal vector (KI construct RRM1, TR#4 R10) by recombineering (Gene Bridges). The linearized vector was electroporated into mES cells by the Transgenic Mice Unit of CNIO. Properly recombined mES cells were identified by Southern Blot and subsequently used for the generation of chimaeric mice. Knock-in mice were genotyped by PCR as described below. To remove the Neo-resistance cassette from the genome, mice were crossed with constitutive CAG-Flpe transgenic mice (Rodriguez et al., 2000). Mice were kept under standard conditions at the specific-pathogen free facility of the Spanish National Cancer Research Centre in a mixed C57BL/6-129/Sv background. All mouse work was performed in accordance with the Guidelines for Humane Endpoints for Animals Used in Biomedical Research, and under the supervision of the Ethics Committee for Animal Research of the "Instituto de Salud Carlos III".

Animal genotyping was performed using DNA extracted from small tail pieces that were digested for 16 hours at 55°C in lysis buffer: 100 mM NaCl, 20 mM Tris-HCl (pH8), 10 mM EDTA, 0.5% SDS and 400 µg/ml (Roche) 400µg/ml. Cellular lysates were treated with a saturated NaCl solution and DNA was precipitated with isopropanol, washed with 70% ethanol and resuspended in distilled water. Genotyping PCRs were performed in a reaction mix composed of 200 µM dNTPs, 1.5 mM $MgCl_2$, 2µl reaction buffer 10X, 0.15 µl Taq polymerase (Platinum Taq, Invitrogen), 0.5 µM of each oligonucleotide and 100ng of genomic DNA.

PCR oligonucleotides:

Rrm1$^{WG(Neo)}$: a band of 135bp for the wt allele and a band of 231bp for the *Rrm1*$^{WG(Neo)}$ allele are expected.

Fwd: GGCCAGCTTGGCAACTTA

Rev (Neo): TGGATGTGGAATGTGTGC

Rev (wt): GAAGTACTGAGATAAACTCC

Rrm1^{WG}: a band of 231bp for the wt allele and a band of 343bp for the *Rrm1*^{WG} allele are expected.

Fwd: GGCCAGCTTGGCAACTTA

Rev: TGCAGTGACTTAGACATCC

1.2 Southern blot

Approximately 15 µg of DNA were digested with the MunI (New England Biolabs) enzyme for 12 hours at 37°C in a solution containing the digestion buffer provided by the manufacturer, 0.1 mg/ml BSA and 2.5 mM spermidine. Digested material was separated by electrophoresis in a 0.8% agarose gel at low voltage until DNA was properly separated. The gel was incubated in a 0.25 M HCl solution for 15 minutes in order to denature the DNA. The gel was treated with 0.4 M NaOH and 0.6 M NaCl and immersed in 0.5 M Tris-HCl (pH7.5), 1.5 M NaCl neutralizing solution for 30 minutes. All treatments were done at room temperature with gentle shaking. DNA was transferred by diffusion in 10XSSC (1.5 M NaCl, 0.15 M $Na_3C_6H_5O_7$) buffer to a positively charged nylon membrane (Hybond XL, Amersham, Buckinghanshire, UK) over night and ultraviolet light was used to enhance covalent linkage of DNA to the membrane (Stratalinker, Stratagene, Agilent Technologies, Santa Clara, CA). The membrane was prehybridized with a hybridizing solution (0.25 M sodium phosphate (pH7.2), 1 mM EDTA, 1% BSA, 7%

SDS) at 65°C for a minimum of 2 hours. During this time radioactive labeling of the probe was performed. 20 ng of the probe were dissolved in a final volume of 45 µl TE. The probe was denaturalized at 99°C and amplified according to the instructions of the Random Prime System (Stratagene) with 50 µCi of [32^{P}] dCTP and purified by filtration in a Sephadex G-50 Column (ProbeQuant GE Healthcare). The membrane-transferred DNA was hybridized to the probe at 65°C overnight in a hybridizing solution containing 0.05 mg/ml salmon sperm (Invitrogen). The membrane was washed in SSC 2x for 10 minutes, in a SSC 2X, 1% SDS containing solution for 30 minutes and finally for another 15 minutes in SSC 0.2X, 0,1% SDS. After the washing steps, the membrane was exposed in a cassette to a PhosphorImager detection screen for the adequate time and developed with a Typhoon TRIO scanner (GE Healthcare).

1.3 Blood Analysis

Blood samples were obtained from the sublingual vein and the blood was collected in EDTA-treated microtubes (Aquisel). These samples were run on the Abacus Junior Vet hematology analyzer (Diatron), providing complete blood counts including counts of white blood cells, red blood cells and platelets.

1.4 Irradiation

Sublethal irradiation (6 Gy of total body ionizing irradiation) was administered (RADSource 2000, X-ray biological irradiator, 160 kV, 4.2 kW, 25 mA) to 8 week old mice. The hematologic parameters were then evaluated at 1-5 weeks post irradiation.

2 Cellular biology

2.1. Cell culture

Unless otherwise specified, all cell lines were cultured in DMEM media (4.5 g/L Glucose; L-Glutamine) (Lonza, Switzerland) with 10% -15% of inactivated fetal bovine serum (FBS) (South American Origin, Lonza) and a mix of penicillin and streptomycin (Gibco, Invitrogen, Life Technologies, Carlsbad, CA). Cells were kept in incubators at 37°C and 5% $C0_2$, except for MEFs, that were maintained in hypoxia incubators, at 37°C and 5% $C0_2$ and 5% O_2.

2.2 Production of MEFs

Female and male mice of the desired genotype were mated until vaginal plugs were visible. At 13.5dpc of gestation the female was sacrificed and embryos were extracted. The fetal liver was removed in sterile conditions, as well as a little piece of the head from which DNA was obtained for genotyping. The remaining embryo was cut with a sterile blade and incubated 10 minutes in 1ml trypsin 0,25%, EDTA (Gibco). The resulting mixture was pipetted up and down in order to dissolve all aggregates and neutralized with 9ml cell culture media. The total 10ml were transferred to a p100 plate that was kept in hypoxia incubators. The media was changed the following day in order to eliminate dead and blood cells.

2.3 Isolation of splenic B lymphocytes

Spleenectomy was performed in mice at age 6 to 10 weeks. Whole spleens were squeezed in washing buffer: PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) with 1% bovine serum albumin (BSA Fraction V, Roche). Supernatants were treated with a hypotonic solution (ACK Lysing Buffer, Lonza) for 5 minutes, after which the solution was inactivated with washing buffer. Cellular aggregates were subsequently eliminated with a 40 μm filter and the lysate was centrifuged 5 minutes at 350g. The pellet was suspended in 900 μl of washing solution containing 80 μl of anti-CD19 antibody conjugated magnetic Mouse CD19 Micro Beads (Miltenyi Biotech, Germany) and incubated at 4°C for 15 minutes. Cells were washed, suspended in 1 ml washing solution and transferred to a separating column (MS Columns, Miltenyi) standing in a magnetic scaffold

(OctoMACS separator, Miltenyi). Cells linked to the anti-CD19 beads were separated using a magnet (model, brand). After washing the column, these cells were eluted outside of the magnetic field. Primary B lymphocytes were maintained in culture (1x 10^6 cells/ml) in RPMI media (Euroclone) supplemented with 10% FBS, 1% penicillin/streptomycin (Gibco, Invitrogen), 2 mM glutamine (Gibco, Invitrogen), non essential amino acids (Lonza), sodium pyruvate (Gibco, Invitrogen), 50 mM β-mercaptoethanol (Gibco, Invitrogen) and 10 mM HEPES (Lonza). 25 mg/ml LPS (LPS, Sigma-Aldrich, St. Louis, MO) was added to stimulate cells.

2.4 Proliferation curve

1.25 x 10^5 MEFs were seeded in a 35mm plate, after 2 days cells were counted and 1.25 x10^5 MEFs were seeded again. The increase in population doublings (PDLs) was calculated applying the formula PDLs = log (nf / n0) / log2, where n0 is the initial cell number and nf is the final cell number in each passage.

2.5 Cell cycle analysis

5x10^5- 10^6 cells were harvested as usual; they were fixed with cold PBS 70% ethanol over night. Cells were resuspended in a PBS solution containing 1% BSA, 10 µg/ml propidium iodide, and 0.5 mg/ml RNase A and were analyzed by flow cytometry in a FACS Calibur machine (BD).

2.6 Gene silencing

Mission endoribonuclease prepared siRNA (esiRNA, Sigma) is a mixture of siRNA oligos resulting from cleavage of long double-stranded RNA with dicer. Transfection of the esiRNA library or siRNAs targeting human KPNB1 (#1 5´-CAGUGUAGUUGUUCGAGAUtt, #2 5´-CAUCCGAUAGAAUCCAGUUtt, 3# 5´-CAGCAAG UUUUAUGCGAAtt, Ambion) was performed using Lipofectamine RNAimax (Invitrogen) according to the manufacturer's instructions.

2.7 Protein expression

Transfection for protein overexpression and viral production were performed with Lipofectamine 2000 (Invitrogen), according to the manufacturers standard protocols. Viral particles for transduction of human RRM2 or human KPNB1 were produced in 293T cells, transfected with a second generation lentiviral platform based on the psPax2 pMDEG packaging vectors. At 48 hours post transfection, the supernatant from the packaging lines was collected, filtered and added on top of experimental cells.

Stable cell lines of Flp-In T-REx 293 cells (Life Technologies) were generated according to the manufacturers instructions. Recombinant protein expression was induced by addition of 100 ng/ml doxycycline to the media for the indicated times. For the determination of protein half-life, cells were incubated with 25 μg/ml cycloheximide (Sigma) for 6/8/10 hours.

In addition, RRM2, RRM2B, RRM1 and RRM1-WG were expressed in *E. coli* BL21 (DE3) (Lifetechnologies). Cells were grown in TB medium at 37°C until the culture reached an OD600 of 0.6. Cells were then chilled to 15°C for 30 minutes and induced with 0.5 mM IPTG for 18 hours at 15°C while shaking at 220RPM. Cells were harvested by centrifugation at 5000 x g for 30 minutes, and cell pellets were stored at -80°C.

2.8 Immunofluorescence/High throughput microscopy (HTM)

Cells were cultured in 96 well plates with a flat crystal bottom (Greiner Bio-One) and treated according to the experimental protocol. Cells were fixed with 2% paraformaldehyde in PBS at room temperature for 5 minutes and permeabilized with 0.1% sodium citrate and 0.1% Triton X-100 in PBS for 5 minutes. After washing three times with 0.25% BSA, 0.1% Tween20 in PBS, cells were incubated in blocking solution (2.5% BSA, 0.1% Tween20, 10% goat serum) for 30 minutes. The corresponding primary antibody diluted in blocking solution was incubated over night at 4 °C. After washing 3 times the secondary antibody conjugated to a

fluorophore was added for 1 hour at room temperature, washed and nuclei were stained with DAPI.

When EdU (5-ethynyl 2'-deoxyuridin) staining was required, cells were treated with EdU for 30 minutes to 1 hour and fixed with 4 % paraformaldehyde in PBS at room temperature for 5 minutes. The Click-iT Edu Cell proliferation Assay kit (Life Technologies) was used to stain the incorporated nucleoside.

Images were automatically acquired from each well by an Opera High-Content Screening System (Perkin Elmer). A 20 x magnification lens was used and pictures were taken at non-saturating conditions. Images were segmented using the DAPI staining to generate masks matching cell nuclei from which the average γH2AX/EdU signal was calculated. Data were represented with the use of the Prism software (GraphPad Software).

3 Molecular biology and biochemistry

3.1 Plasmid construction

For the construction of pcDNA5/FRT/TO-RRM1 with a C-terminal Strep-tag, the cDNA of hRRM1 was amplified by PCR from human cDNA and cloned into pEXPR-IBA103 (Novagen) vector at SacII/XhoI sites. From there, the RRM1-Strep sequence was PCR amplified adding AflII/NotI restriction sites for subsequent cloning into the pcDNA5/FRT/TO vector (Life Technologies). Expression plasmids for RRM1-WG were constructed by introducing the W684G mutation into the wild-type pEXPR-IBA103 expression plasmid using the Quick Change site-directed mutagenesis kit (Agilent Technologies) followed by PCR and subcloning into the pcDNA5/FRT/TO vector as described above. The final constructs were sequenced to rule out the presence of mutations. The plasmids coding for KPNB1, RRM2 and the empty vector were obtained from the Precision LentiORFTM collection (Dharmacon).

For bacterial expression, the cDNAs of human RRM1, RRM1-WG, RRM2 and RRM2B were cloned into the pET30a expression vector at SalI/NotI (RRM1 and RRM1-WG) or BamHI/XhoI (RRM2 and RRM2B) RS sites and expressed as 6xHistidine-tagged (His-tag) versions in Bl21 cells. In addition, RRM1 and RRM1-WG were expressed as a Streptavidine-tagged (Strep-tag) version by removal of the N-terminal His-tag and introduction of a Strep-tag into the pET30a vector.

3.2 Western blot

The following primary antibodies were used: Strep-Tag II (Novagen, 71590-3), RRM2B (Abcam, ab8105) RRM2 (Santa Cruz, Sc-10844), RRM1 (Cell Signaling, 3388), and β-Actin (Sigma, A5441), CHK1 (Novocastra), CHK1-S345P (Cell Signaling Technology, 2348S), RPA (Abcam, ab2175), RPA-S4P/S8P (S4/S8) (Bethyl, A300-245A) and γH2AX (Upstate, 05-636), KPNB1 (Abcam, ab2811), HIS (Rockland 600-401-382).

For extraction of whole cell lysates, cells were lysed for 10 minutes on ice in RIPA buffer (50 mM Tris-HCl (pH7.4), 1% NP-40, 0.25% Na-deoxycolate, 150 mM NaCl and 1 mM EDTA) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich) and centrifuged at 16000g to eliminate cell debris. Cytosolic and nuclear extracts were prepared as previously described (Lecona et al., 2008). Bradford method was used to determine protein concentration. Extracts were denatured in NuPage loading buffer (Lifetechnologies) heated for 10 minutes at 75°C, and separated in gradient gels 4-12% SDS-PAGE (Tris-Acetate Nupage Novex, Invitrogen). Proteins were subsequently wet-transferred to nitrocellulose membranes (Hybond ECL Nitrocellulose, Amersham). After transfer, membranes were incubated for 30 minutes at room temperature in blocking solution (5% skimmed milk, Central Lechera Asturiana) in 0.1% TBS-Tween20 (TBS-T). Membranes were incubated overnight at 4°C with primary antibodies diluted in TBS-T 5% BSA. After washing the membranes 3 times with TBS-T, they were incubated for 1 hour at room temperature with HRP-conjugated secondary AB. Proteins were visualized with SuperSignal West Pico or Femto Chemiluminescent

Substrate (Lifetechnologies) on a ChemiDoc XRS Gel Photo Documentation System (Bio-Rad).

3.3 Immunoprecipitation

For protein Immunoprecipitation from Flp-In T-REx 293 cells, cells were washed once with PBS, and lysed in RIPA buffer (50 mM Tris-HCl (pH7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing protease and phosphatase inhibitors (Sigma) followed by centrifugation at 16000xg for 30 minutes to produce clear lysate. The supernatant was loaded on a Micro Bio-Spin™ Chromatography Columns (Bio-Rad) and incubated with Strep-Tactin Macroprep Resins (Iba) for 2 hours at 4°C on a rotator. The column was washed 5 times with buffer W (100 mM Tris-HCl (pH8), 1 mM EDTA, 200 mM NaCl) containing 0.1% NP-40. RRM1 or RRM1WG and associated proteins were eluted with 2mM Biotin in buffer W.

3.3.1 Recombinant protein purification

For the isolation of recombinant protein from bacteria, cells were thawn on ice and resuspended in washing buffer (50 mM Tris (pH7.8), 200 mM NaCl, 2 mM PMSF and 1 mM dithiothreitol (DTT)).

Cells were ruptured by sonication cycles at 4°C and centrifuged at 30000g for 30 min at 4°C. The supernatant was incubated with Ni-NTA agarose (Qiagen) or Strep-Tactin Macroprep Resins for 1 hr at 4°C on a rotator and loaded onto a biospin disposable chromatography column. For purification of Strep-tagged proteins, beads were washed 3 times with washing buffer and bound protein was eluted with washing buffer containing 2 mM biotin. For the purification of His-tagged proteins, 10 mM imidazole was added to the washing buffer and samples were eluted in buffer containing 250 mM imidazole.

Fractions were analyzed for purity using a 12% SDS-PAGE gel and coomassie staining. Fractions containing RRM1, RRM1-WG, RRM2 or RRM2B were pooled and

dialysed against 50 mM Tris (pH7.8), containing 200 mM NaCl, 2.5 mM, 2 mM PMSF, 10% glycerol and purified proteins were stored at -80°C.

3.4 In-vitro binding assay

Generally, 1,5µg Strep-RRM1 or Strep-RRM1-WG and 1,5µg of indicated His-RNR subunits were mixed in 0,5 ml of IP buffer (25 mM Tris-HCl (pH7,5), 200 mM NaCl, 1 mM EDTA, 2 mM PMSF, 1 mM DTT) and incubated for 20 minutes at RT. Strep-tactin beads were added and samples incubated for 1.5 hours under constant rotation at 4°C. Beads were washed 5 times and bound proteins were eluted in IP buffer containing 2 mM biotin. Proteins were separated on a SDS gel and stained with coomassie or analyzed by WB.

3.5 RNA extraction and analysis

Total RNA was isolated using Absolutely RNA Microprep Kits (Agilent) according to manufacturers recommendations. Samples harvested at different time points were stored in lysis buffer at -80°C and extraction was performed at the same time for all samples. After the extraction, samples were stored at -80°C.

cDNA was synthesized using the SuperScript II Reverse Transcriptase kit for RT–PCR (Invitrogen). Real-time PCR was performed using the SYBR-Greener qPCR Supermix (Invitrogen) in the Cycler Real Time PCR System (BioRad). GAPDH expression level was used to normalize values of gene expression. Data are shown as fold change relative to the sample control and at least two independent experiments in triplicate were performed. The oligonucleotides used were:

GAPDH Fwd: GCCACCCAGAAGACTGTGGATGGC

GAPDH Rev: CATGATGGCCATGAGGTCCACCAC

RRM1 Fwd: TGTGGATCAAGGTGGAAACA
RRM1 Rev: GGGATCCAAAGTGCAAAGAA

RRM2 Fwd: CCTACTAACCCCAGCGTTGA

RRM2 Rev: GTTTCAGAGCTTCCCAGTGC

RRM2B Fwd: TACAAGCAAGCACAGGCATC

RRM2B Rev: CGCTCCACCAAGTTTTCATT

3.6 dNTP measurements

For LC-MS/MS analysis of cytosolic fractions the instrument consisted of an Acquity Ultra Performance LC and a Xevo TQ using an ESI source operated in negative mode (Waters, Sollentuna, Sweden). 200 µl of 60% acetonitrile in water containing 100 mM ammonium acetate and 10 mM ammonium phosphate was added to five million cells followed by 10 minutes of sonication using an Ultrasonic Cleaner 5510 (Branson Ultrasonics, Danbury, CT) to lyse cell membranes and precipitate the proteins. After centrifugation, 20 µl of the supernatant was injected on a ZIC-cHILIC column (100x2.1 mm, 3 µm, Merck-Millipore, Sollentuna, Sweden) termostated at 30 °C. dNTPs were separated using a six minute gradient from 65 to 50% acetonitrile at a flow rate of 400 µl/min.

For DNA Polymerase assays two million MEF were resuspended in 60% methanol, frozen in liquid nitrogen and boiled for 3 minutes. Samples were evaporated until dryness in a speedvac and whole cell levels of dNTPs were determined using the DNA polymerase assay previously described (Desler et al., 2007).

RESULTS

1 Initial evidences for a connection between ATR and the RNR

ATR, like its yeast ortholog Mec1, is essential for cell viability since it is the main kinase controlling checkpoint signaling during the cell cycle and in response to DNA damage (Elledge, 1996; Weinert, 1998). In contrast to ATR, the essential function of Mec1 is well understood and consists in the positive regulation of the dNTP pool through induction of RNR activity. In *S.cerevisiae*, Mec1 activation leads to phosphorylation and subsequent degradation of the RNR inhibitor Sml1, resulting in an increase of RNR activity and dNTP levels. Importantly, deletion of Sml1 rescues the viability of *mec1Δ* mutants, strongly suggesting that the essential role of Mec1 is related to RNR activity (Zhao et al., 2001; Zhao and Rothstein, 2002). Whereas RNR subunits are highly conserved from yeast to humans, including the sequence surrounding the binding site of Sml1 to Rnr1, no allosteric inhibitors of the canonical RRM1-RRM2 complex have been described in mammals. However, several evidences suggest that the ATR-dependent regulation of the RNR could be to some extent conserved in mammals. First, addition of nucleosides has been shown to reduce oncogene-induced RS in mammalian cells (Bester et al., 2011). Second, a recent paper directly linked ATR signaling to RRM2 stability (D'Angiolella et al., 2012). Third, the transcriptional regulation of RNR genes by ATR is similar in mammals and yeast. Here, we wanted to explore to what extent the ATR-dependent control of the RNR and dNTP pools is conserved in mammals.

While *Rrm2* expression has been directly linked to ATR signaling (Zhang et al., 2009), *Rrm1* expression had only been linked to ATM activity, the main checkpoint kinase responsible for signaling DNA DSBs (Eaton et al., 2007). To explore in detail the impact of ATR signaling on the transcription of the different RNR subunits, we measured their mRNA expression levels in response to conditions that either induce or inhibit ATR signaling. On one hand, the RS checkpoint was abrogated

using chemical inhibitors of ATR (Toledo et al., 2011) or CHK1 (UCN-01). On the other hand, ATR signaling was induced by the usage of HU, an inhibitor of the RNR that quickly induces RS by nucleotide depletion and hence activates ATR signaling. In addition, we took advantage of a cellular system (3T3-TopBPI-ER) in which ATR can be activated in response to tamoxifen (4-OHT), and in the absence of any actual DNA damage (Toledo et al., 2008). Expression of *Rrm1*, *Rrm2* and *Rrm2b* were induced in response to HU, while inhibition of CHK1 repressed *Rrm1* and *Rrm2* expression **(Figure 8A)**. In contrast, *Rrm2b* was induced in response to CHK1 inhibition, potentially caused by CHK1i-induced DNA breaks. Importantly, inhibition of ATR or CHK1 repressed *Rrm1* expression while its activation induced *Rrm1* **(Figure 8B)**. Our results show a clear correlation between ATR activity and expression of both RNR subunits involved in DNA replication, *Rrm1* and *Rrm2.* Together our data indicate that ATR indeed regulates not only transcription of *Rrm2* but also of *Rrm1*, confirming a link between ATR and the RNR in mammals.

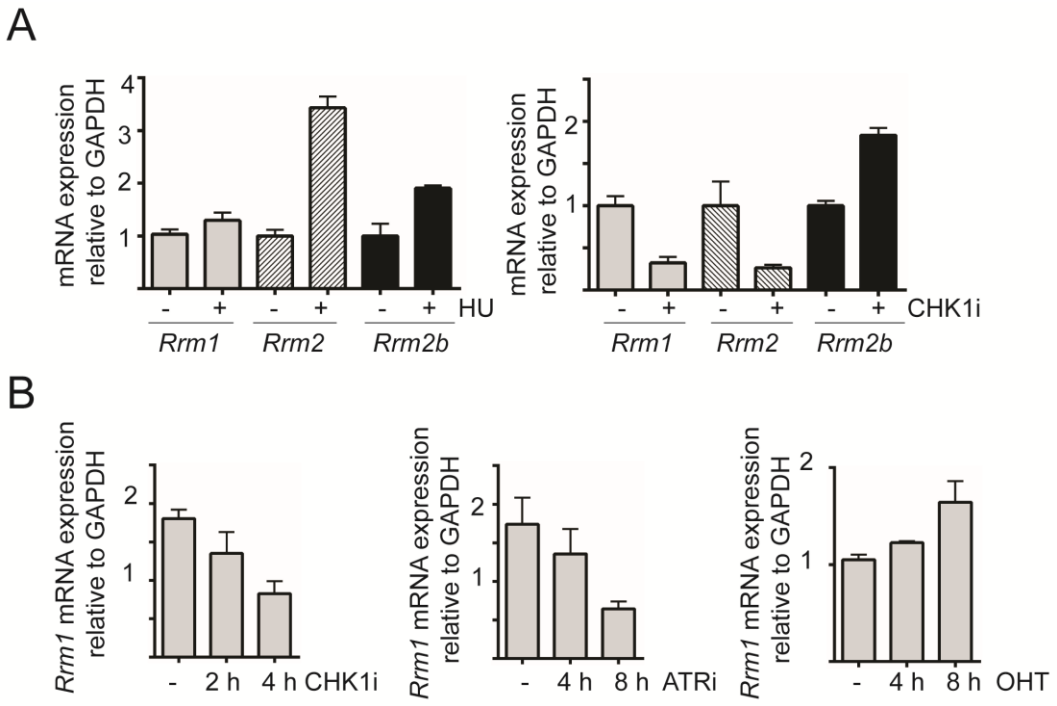


Figure 8: ATR regulates the expression of the RNR subunits *Rrm1, Rrm2* and *Rrm2b*. (A) Expression of *Rrm1*, *Rrm2* and *Rrm2b* were induced in response to HU, while inhibition of CHK1 (CHK1i) repressed *Rrm1* and *Rrm2* expression but induced *Rrm2b*. Cells were treated where indicated for 8 hours with 2mM HU or 300nM UCN-01 (CHK1i). (B) ATR was activated upon

tamoxifen (OHT) treatment (Toledo *et al*., 2008) and induced *Rrm1* expression while its inhibition, as well as inhibition of CHK1, repressed *Rrm1*.

Interestingly, the results were further supported by available data from our lab that showed a correlation between *Rrm2* expression and ATR in mice. The murine model for the ATR-Seckel Syndrome previously developed in our lab (Murga et al., 2009) presents severely reduced ATR activity resulting in increased RS, stunted growth and premature aging (Monasor et al., 2013; Murga et al., 2009). Microarray data from this study revealed reduced levels of *Rrm2* expression in ATR mutant embryonic livers and brains, indicating that low *Rrm2* expression might contribute to the ATR-Seckel phenotype (Murga et al., 2009). Taken together, our results point towards a connection between ATR function and RNR activity in mammals and prompted us to explore whether increasing RRM2 expression could alleviate the physiological consequences of reduced ATR activity in mice.

2 Increased Rrm2 gene dosage reduces RS and prolongs survival of ATR deficient mice

2.1 Nucleosides limit RS and improve growth of ATR-Seckel MEFs

As mentioned before, early studies in yeast had shown that Mec1 deficiency could be rescued by situations that increase cellular dNTP levels, such as overexpression of RNR or exogenous supply of nucleotides, indicating that stimulation of dNTP production is an essential function of Mec1. Along these lines, a recent study also shows that oncogene-induced RS can be reduced by exogenous supply of nucleotide precursors in mammalian cells (Bester et al., 2011). To determine whether a reduced pool of dNTPs could underly the accumulation of RS and growth defects of ATR-Seckel cells, we first tested the effect of an exogenous supply of nucleosides. RS levels can be rigorously quantified via high-throughput microscopy (HTM) by measuring ATR-dependent H2AX phosphorylation (γH2AX) (Murga et al., 2011; Toledo et al., 2011). Our results showed that an extra supply of nucleosides significantly reduced levels of RS in ATR-Seckel MEF and partially recovered their growth rates **(Figure 9).**

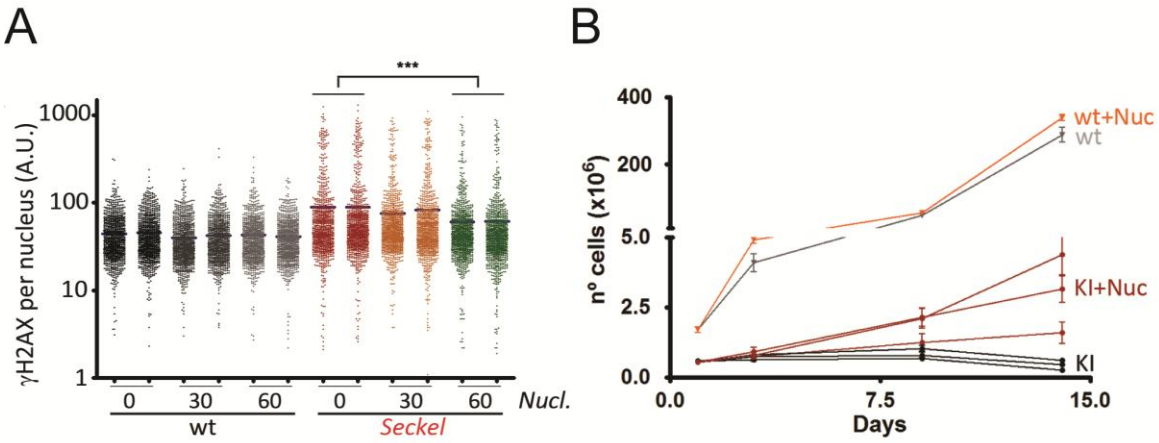


Figure 9: Nucleoside supplementation reduces RS and improves growth of ATR-Seckel MEF. (A) HTM-mediated quantification of the intensity of γH2AX per individual nucleus on *Atr*$^{+/+}$ and *Atr*$^{S/S}$ MEF treated in the absence or presence of nucleosides (60μM) for 24 hours. Data are representative of 2 independent cell lines. (B) Proliferation curves of *Atr*$^{+/+}$ and *Atr*$^{S/S}$ MEF grown in the presence or absence of nucleoside supplementation (60μM). Data are representative of two independent analyses (Lopez-Contreras et al 2015).

These initial studies suggested that increased levels of nucleotides could potentially suppress the physiological consequences of reduced ATR activity in mammals.

2.2 RRM2 overexpression protects from reduced ATR activity in vitro and in vivo

We next explored whether genetic manipulations that stimulate RNR activity could also rescue phenotypes linked to limited ATR activity in mammals. To this end, we generated a U2OS cell line stably overexpressing RRM2. We selected RRM2 for the following reasons: first, RRM2 is rate limiting for mammalian RNR activity (Lewis and Wright, 1974; Tonin et al., 1987); second, RRM2 is stabilized in response to ATR signaling after DNA damage (D'Angiolella et al., 2012); finally, the previously mentioned microarray data from our lab pointed towards a potential correlation between reduced *Rrm2* expression and phenotypes caused by ATR deficiency (Murga et al., 2009). To test whether RRM2 overexpressing cells exhibit increased RNR activity, we examined their response to the RNR inhibitor HU. HTM analyses revealed that RRM2 overexpressing cells exhibited lower levels of γH2AX and phosphorylation of RPA and CHK1 in response to HU **(Figure 10A)** while cell replication was not altered **(Figure 10B)**, confirming an increased RNR activity in those cells. Depletion of the RNR subunits had the opposite effect **(Figure 10C,D)**. Importantly, RRM2 overexpressing cells also exhibited reduced levels of γH2AX in response to ATR or CHK1 inhibitors **(Figure 10A).** Together, these results show that overexpression of RRM2 leads to enhanced RNR activity in mammalian cells and that this confers increased resistance to the replicative damage induced by limited ATR/CHK1 signaling.

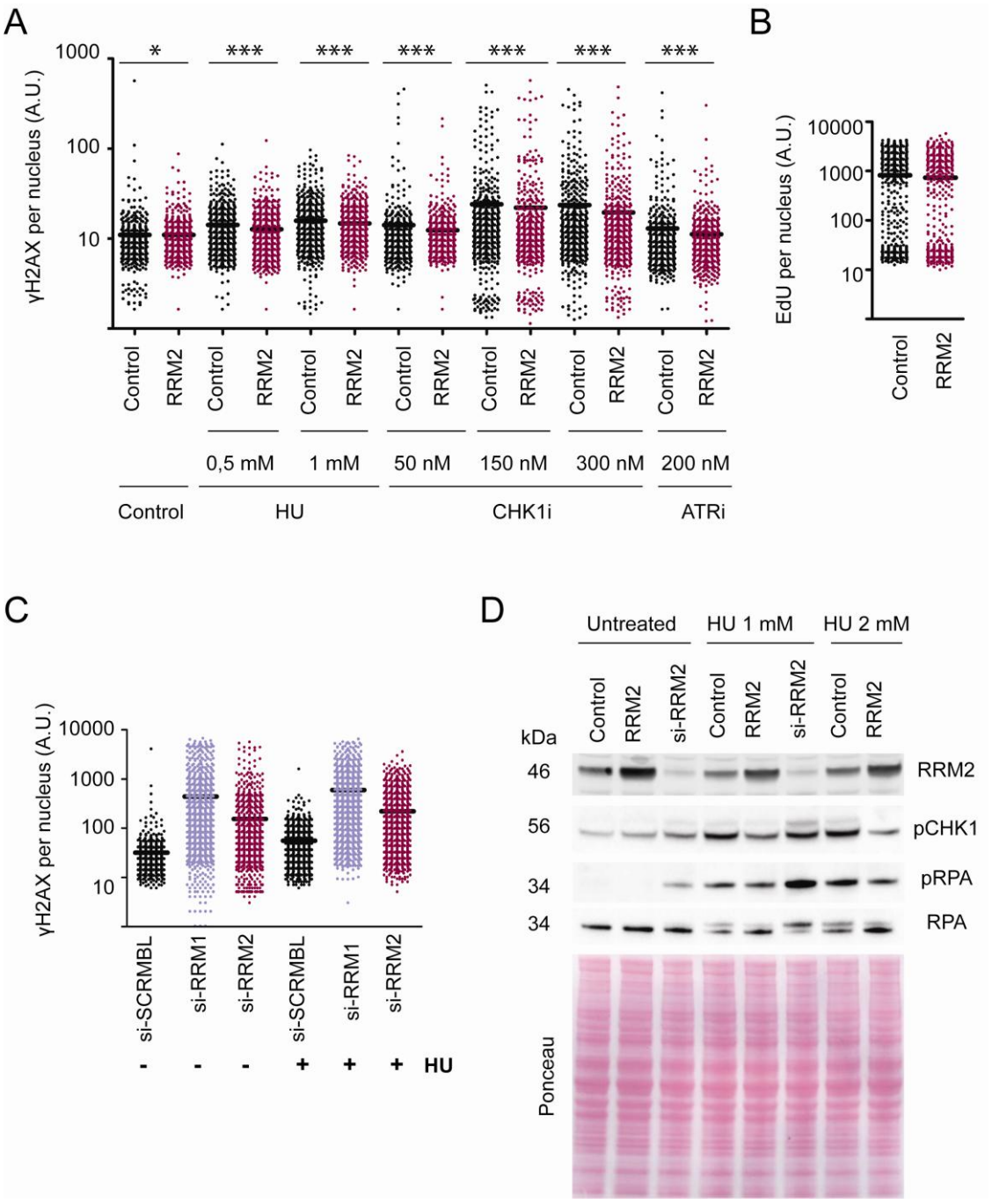


Figure 10: RRM2 overexpression reduces RS levels. (A) HTM-mediated quantification of the intensity of γH2AX per individual nucleus on human U2OS cells overexpressing RRM2, or the empty vector (Control). Where indicated, cells were also exposed to the indicated concentrations of HU, CHK1i or ATRi for 3 hours. (B) HTM-mediated quantification of EdU uptake. Cells were labeled with EdU for 1 hour. (C) HTM-mediated quantification of the intensity of γH2AX per individual nucleus on human U2OS cells 48 hrs after being transfected with either a scrambled control esiRNA (Control) or esiRNAs targeting RRM1 or RRM2. Where indicated, cells were also exposed to HU (2mM) for 3 hours. Data is representative of three independent experiments. (D) Western blot

illustrating the impact of RRM2 overexpression (RRM2) or depletion (si-RRM2) on RPA and CHK1 phosphorylation in U2OS cells. Ponceau is shown as a loading control. HU was added for 3 hours where indicated.

Given that we observed increased resistance towards ATR inhibitors in cells with elevated RNR activity, we explored the effects of ATR inhibition on cellular dNTP levels. To this end we measured dNTP levels in NIH 3T3 cells after exposure to HU or ATR inhibitors. Remarkably, two independent methods of dNTP quantification revealed that ATR inhibition leads to a reduction in cellular dNTP levels **(Figure 11A,B).** The greater effect of HU on dATP and its impact on dTTP levels is consistent with previous literature (Julias and Pathak, 1998). These results reveal that an exhausted dNTP pool could contribute to the toxicity of ATR inhibitors.

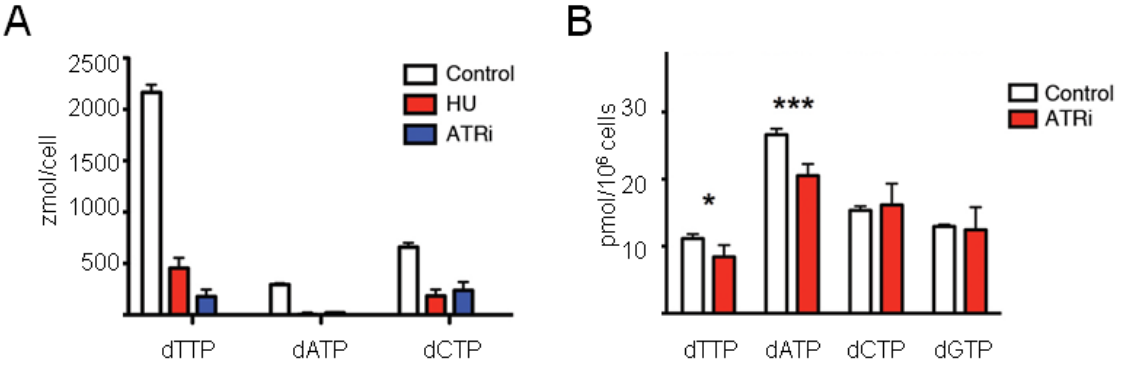


Figure 11: Nucleotide concentrations in response to ATR inhibition. dNTP levels were measured by two independent methods: liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)(A) or a DNA polymerase assay. (A) LC-MS analysis represents cytosolic dNTP levels and (B) DNA Polymerase assays whole cell levels. Analyses were performed on NIH 3T3 cells exposed to HU (2mM, 6 hours) or ATRi (1μM, 6 hours). In (A), dGTP levels were undetectable. Each figure depicts one representative analysis where biological triplicats were analysed in parallel (Lopez-Contreras et al., 2015).

Finally, after confirming that increased RRM2 levels reduced the toxicity of ATR inhibitors, we sought to investigate the consequences of increased RNR activity *in vivo.* To this end, Andres López-Contreras in the laboratory generated mice carrying an extra allele of the RRM2 subunit (*Rrm2*^{TG}). In agreement with our results obtained in U2OS cells, *Rrm2* transgenic cells showed increased RNR activity and an increased resistance towards ATR inhibitors (Lopez-Contreras et

al., 2015). When crossed with ATR-Seckel mice, the presence of the *Rrm2* transgene significantly reduced the ATR-Seckel associated phenotypes such as reduced body size and craniofacial abnormalities. Importantly, ATR-hypomorphic mice carrying an extra allele of *Rrm2* doubled the median life span of their littermates lacking the *Rrm2* transgene (50 weeks vs. 24 weeks) and increased the maximum life span from 54 weeks to 91 weeks **(Figure 12).** Taken together, our results show that, similar to yeast, ATR deficiency can be partially rescued by increased RNR activity in mammals (Lopez-Contreras et al., 2015).

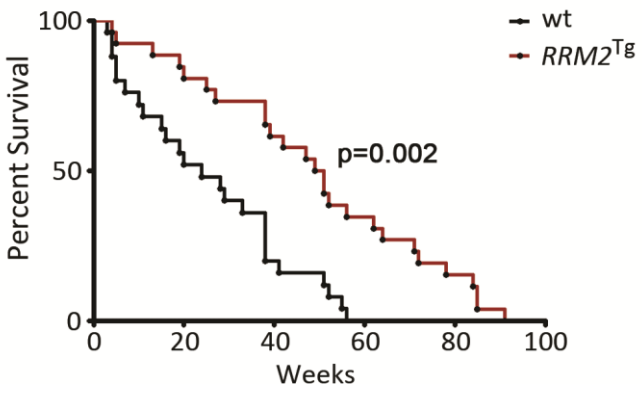


Figure 12: Kaplan-Meier curves of *ATRS/S;Rrm2+/+* (n = 25) and *ATRS/S; Rrm2+/TG* (n = 26) mice. The p value was calculated with the Mantel-Cox log rank test (Lopez-Contreras et al., 2015).

In conclusion, we have shown that overexpression of the RRM2 subunit leads to increased RNR activity in mammals and it confers resistance towards replicative damage induced by ATR deficiency. Furthermore, our results indicate that part of the toxicity caused by ATR inhibitors might be due to reduced dNTP levels. Together, these results point towards a conservation of the ATR-dependent regulation of the dNTP pool through regulation of RNR in mammals.

3 Exploring new interactors for the RNR

As described, RNR is an evolutionary well conserved, highly regulated protein complex whose activity is controlled by Mec1, allosteric inhibitors, and nuclear sequestration in fission and budding yeast **(see Introduction 2.2.2)**. Given that the post-transcriptional regulation of mammalian RNR remains poorly defined, we decided to search for new interactors of the mammalian RNR by MS. We employed various experimental approaches in order to purify the different RNR subunits as well as the whole RNR complex. First, we generated constructs comprising human RRM1 or RRM2 with a C-terminal Twin-Streptavidine-tag (Strep-tag) that were expressed in the FlpIn T-REx 293 cell line, a system allowing for homogeneous and doxycycline-inducible expression of a given cDNA from a single integration site **(Figure 13)**.

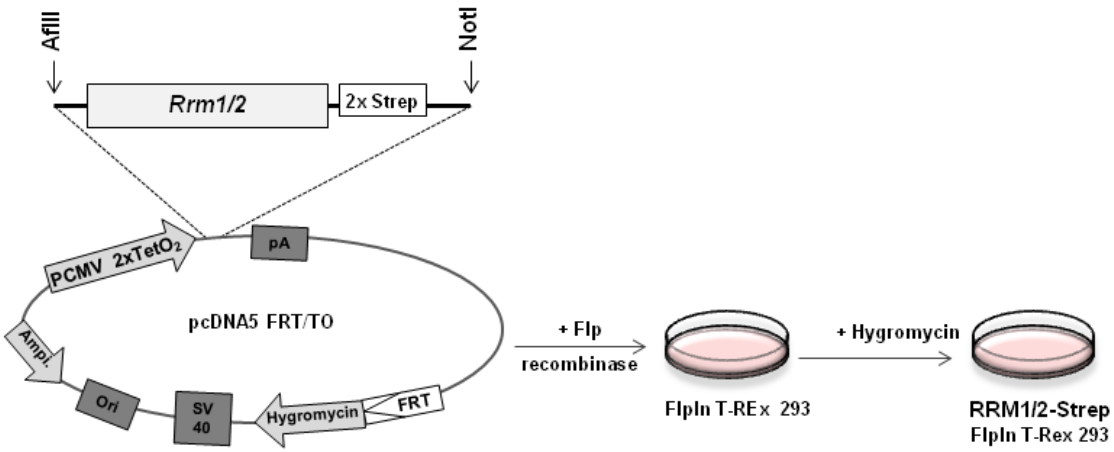


Figure 13: Generation of RRM1 or RRM2 overexpressing cell lines. The human cDNA of RRM1 or RRM2 was cloned upstream of a 2 x Strep-tag into the pcDNA5-FRT/TO expression vector. Transfection of the pcDNA5-FRT/TO construct in parallel with the FLP recombinase leads to integration into a single site in the genome of the FlpIn T-REx 293 cell line. A stable cell line carrying the pcDNA5-FRT/TO-RRM1/2 plasmid was selected with hygromycin. Expression of the construct is repressed by the Tet repressor in the absence of the inducer doxycycline.

In order to identify proteins that interact with the RNR subunits, we performed Strep IP from lysates of the RRM1-Strep or RRM2-Strep FlpIn T-Rex 293 cells and, with the help of the Proteomics Unit of CNIO, analyzed samples by mass spectrometry (MS). As expected, RRM1 was the protein identified with the highest score after RRM1-Strep pull-down, followed by RRM2 and RRM2B. However, MS data also revealed an about 30 fold enrichment of the purified RRM1-Strep peptides over its RNR complex binding partners, most likely due to the high overexpression of the recombinant protein. This brought us to speculate, that the overexpression of only one subunit might yield physiologically irrelevant results since free RRM1-Strep or RRM2-Strep rather than the active complex might be purified. On the basis of the fact that we were looking for proteins interacting with and regulating the activity of the RNR holocomplex, we aimed to improve the method in order to purify the entire RNR complex. To this end, we designed an expression vector, where we introduced an internal ribosomal entry site (IRES) followed by an N-terminally Flag-tagged RRM2 behind the RRM1-Strep sequence **(Figure 14A)**. This approach allowed the expression of similar levels of RRM1-Strep and Flag-RRM2, and the purification of the whole complex by serial Strep-Flag-IP **(Figure 14B)**. The complex was expressed and purified in a ratio of 1:2,5 for RRM1:RRM2, indicating that we enriched for the physiological RNR complex **(Figure 14C,D)**. We followed a similar approach to purify the alternative RNR complex, RRM1-RRM2B. MS analysis showed that the RRM1-RRM2B complex could also be efficiently purified **(Figure 14E)**. In all cases we could detect the co-precipitation of the alternative small subunit (RRM2B and RRM2 for pull-down of RRM1-RRM2 and RRM1-RRM2B respectively) indicating that the method worked reliably. Unfortunately, looking at the ensemble of our experiments, no consistent and common new protein interactor of the RNR could be identified.

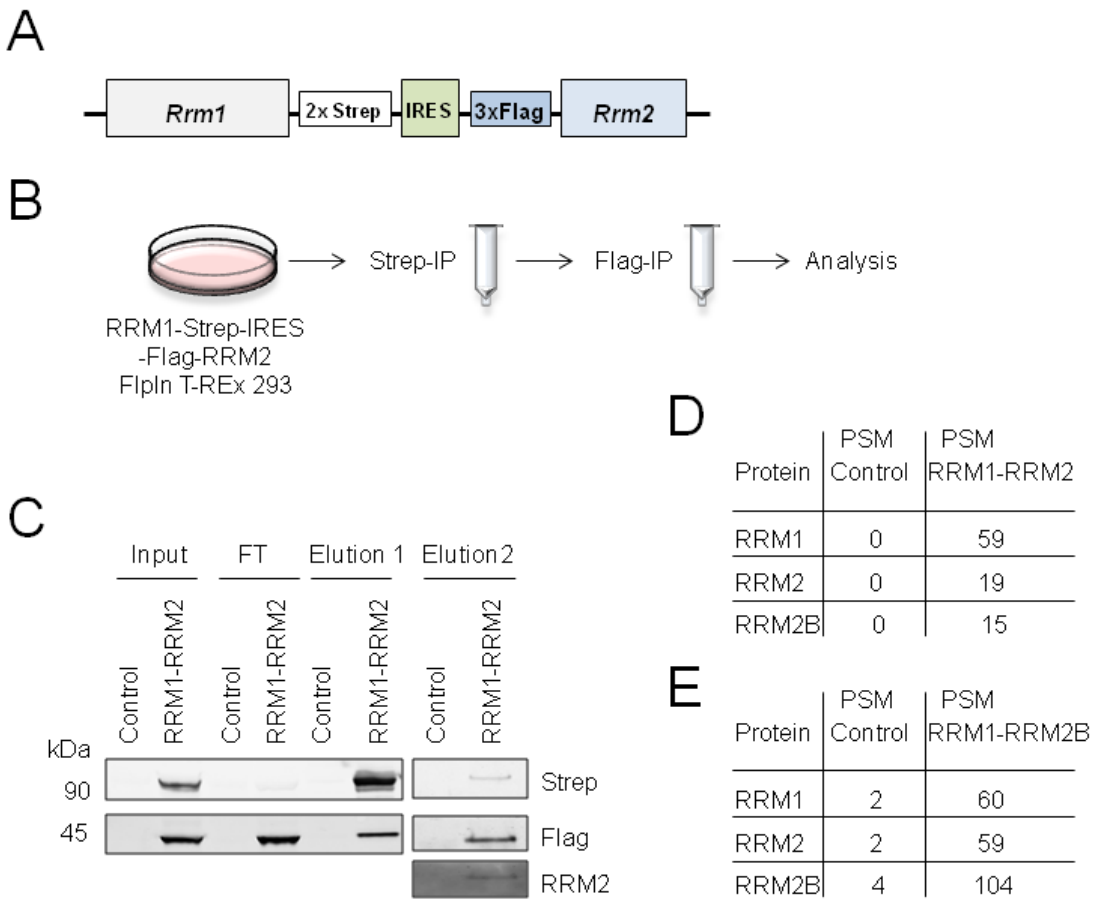


Figure 14: Purification of the RNR complex. (A) pcDNA5-FRT/TO expression construct comprising the C-terminally tagged human *Rrm1* sequence followed by an internal ribosomal entry site (IRES) and the Flag-tagged human *Rrm2* sequence. (B) Experimental pipeline for the purification of the RNR complex from the RRM1-Strep-Flag-RRM2 expressing FlpIn T-REx cell line. (C) WB analysis of immunoprecipitated RNR complex. FT = flow through, Elution 1 = pull down of Strep-RRM1, Elution 2 = pull down of Flag-RRM2. (D) Mass spectrometry data showing the purification of the RRM1-RRM2 complex. (E) Mass spectrometry data showing the purification of the RRM1-RRM2B complex. PSM = peptide spectrum match.

3.1 A genetic screening for RNR interactors

Even though no consistent new interaction partners of the RNR could be identified in our pull-down experiments, we reasoned that some of the weak interactors found in our proteomics could be bona-fide regulators of the RNR. In agreement with this, we were able to identify cyclinF, and members of the APC or SCF

ubiquitin ligases in our experiment **(Table 1)**, which are known regulators of RNR levels (Chabes and Thelander, 2000; Chabes et al., 2003b; D'Angiolella et al., 2012). We thus decided to select a number of candidates for a subsequent genetic screening from the list of proteins identified by MS, further exploring their effect on RNR activity. To this end, we subjected the list of potential RNR interactors to an academically informed examination. Based on their described function (e.g. replication, damage response, signaling or nuclear import), we selected 56 candidate genes for further investigation **(Table 1)**.

PSMD2	26S proteasome non-ATPase regulatory subunit 2	cleave peptides in an ATP/ubiquitin-dependent process, may act in TNF signalling pathway
IRS4	Insulin receptor substrate 4	interface between multiple growth factor receptors possessing tyrosine kinase activity
PHRF1	PHD and ring finger domains 1	protein domain specific binding and RNA polymerase binding
PELO	Pelota homolog	may have a role in spermatogenesis, cell cycle control, and in meiotic cell division
C2orf29	chromosome 2 open reading frame 29	role in mRNA decay
NUCB2	nucleobindin 2	suggested role in calcium level maintenance, release of tumor necrosis factor
WDR6	WD repeat-containing protein 6	interacts with serine/threonine kinase 11, cell growth arrest
WDR5	WD repeat-containing protein 5	part of the MLL1/MLL complex, histone modifications
WDR36	WD repeat-containing protein 36	Involved in T-cell activation and highly coregulated with IL2
WDR87	WD repeat-containing protein 87	
CSNK2A1	Casein kinase 2, alpha 1	Serine/threonine kinase
MELK	Maternal embryonic leucine zipper kinase	Serine/threonine-protein kinase, cell cycle regulation, self-renewal of stem cells, apoptosis and splicing regulation
NEK8	Serine/threonine-protein kinase *Nek8*	role in organogenesis, involved in regulation of Hippo signaling pathway
SKP1A	S-phase kinase-associated protein 1A	member of the SCF ubiquitin ligase protein complex
C19orf62	BRISC and BRCA1 A complex member 1	interacts with BRE
FXR1	fragile X mental retardation, autosomal homolog 1	RNA binding protein, shuttles between nucleus and cytoplasm, associates with polyribosomes
NASP	Nuclear Autoantigenic Sperm Protein	H1 histone binding protein, transporting histones into the nucleus of dividing cells
ASCC2	activating signal cointegrator 1 complex subunit 2	Enhances NF-kappa-B, SRF and AP1 transactivation
STUB1	STIP1 Homology And U-Box Containing Protein 1	E3 Ubiquitin protein ligase/cochaperone
TRIM25	Tripartite Motif Containing 25	may act as a transcription factor
LSM7	U6 snRNA-associated Sm-like protein	may act in pre-mRNA splicing
MLF2	Myeloid Leukemia Factor 2	associated with (myeloid) leukemia
STRAP	serine/threonine kinase receptor associated protein	WD40 repeat protein
SART3	squamous cell carcinoma antigen recognized by T cells 3	RNA-binding nuclear protein, tumor-rejection antigen
C2orf47	chromosome 2 open reading frame 47	
MMS19	MMS19 nucleotide excision repair homolog	incorporation of iron-sulfur cluster into apoproteins, DNA metabolism and genomic integrity
MSH6	mutS homolog 6	DNA mismatch repair

ANAPC4	Anaphase promoting Complex Subunit 4	promotes metaphase-anaphase transition by ubiquitinating specific substrates (e.g. RRM2)
CCNF	cyclin F	Substrate recognition component of SCF, E3 ubiquitin-protein ligase complex
DDX10	Probable ATP-dependent RNA helicase DDX10	putative RNA helicase
POT1	Protection of telomeres protein 1	Component of telomerase ribonucleoprotein (RNP) complex
NME7	Nucleoside diphosphate kinase 7	nucleoside diphosphate kinase activity
NME3	Nucleoside diphosphate kinase 3	role in synthesis of nucleoside triphosphates other than ATP
IPO8	Importin 8	nuclear import of proteins with a classical nuclear localization signal
XPO1	exportin 1	leucine-rich nuclear export signal (NES)-dependent protein and RNA transport
KPNB1	Karyopherin beta 1	Nucleocytoplasmic transport
KPNA1	Karyopherin alpha 1	Nucleocytoplasmic transport
MCM3	minichromosome maintenance complex component 3	key component of the pre-replication complex
MCM9	minichromosome maintenance complex component 9	initiation of genome replication, binds, and regulates chromatin licensing and DNA replication factor 1
RFC3	Replication factor C (activator 1) 3	member of accessory protein complex RFC essential during DNA elongation
RFC5	Replication factor C (activator 1) 5	member of accessory protein complex RFC essential during DNA elongation
SIP1	Zinc finger E-box-binding homeobox 2	two-handed zinc finger TF
ZMYM3	zinc finger, MYM-type 3	component of histone deacetylase-containing multiprotein complexes
DPF2	Zinc finger protein ubi-d4	transcription factor necessary for apoptotic response following deprivation of survival factors
SMC4	structural maintenance of chromosomes 4	mitotic chromosome condensation in frogs and for DNA repair in mammals
PDS5A	PDS5 cohesin associated factor A	binds cohesin complex, associates with chromatin, role in regulating sister chromatid cohesion during mitosis
NT5DC3	5'-Nucleotidase Domain-Containing Protein 3	hydrolase activity
GEMIN5	Gem-associated protein 5	role in assemply of snRNPs
ARF3	ADP-ribosylation factor 3	role in protein trafficking, vesicular trafficking, activator of phospholipase D
C1orf25	TRMT1-like protein	SAM-binding methyltransferase
CHD4	chromodomain helicase DNA binding protein 4	member of nucleosome remodeling and deacetylase complex, transcriptional repression
HDAC6	Histone deacetylase 6	histone deacetylase activity, represses transcription

Table 1: List of candidate genes selected based on results obtained from the RNR pull-down experiments, and targeted in the genetic screening. Gene functions selected include but are not limited to DNA replication and repair, transcription factor and nuclear import. Genes known to interact with the RNR are highlighted in red.

Next, we designed a screening approach that would allow to functionally test whether any of the candidate genes could modulate RNR activity in cells. The screening strategy included the targeting of the candidates with Endoribonuclease-prepared siRNAs (esiRNAs) followed by treatment with a low dose of the RNR inhibitor HU. Targeting of a gene that induces or inhibits RNR activity would result in reduced or increased levels of HU-induced γH2AX **(Figure 15A)**. As a proof of principle, when RRM1 or RRM2 were targeted by esiRNAs, their downregulation

led to a significant increase in HU-induced γH2AX **(Figure 15B)**. Following this pipeline, we identified one protein, Karyopherin-beta 1 (KPNB1), the depletion of which severely induced RS in response to RNR inhibition **(Figure 15B).**

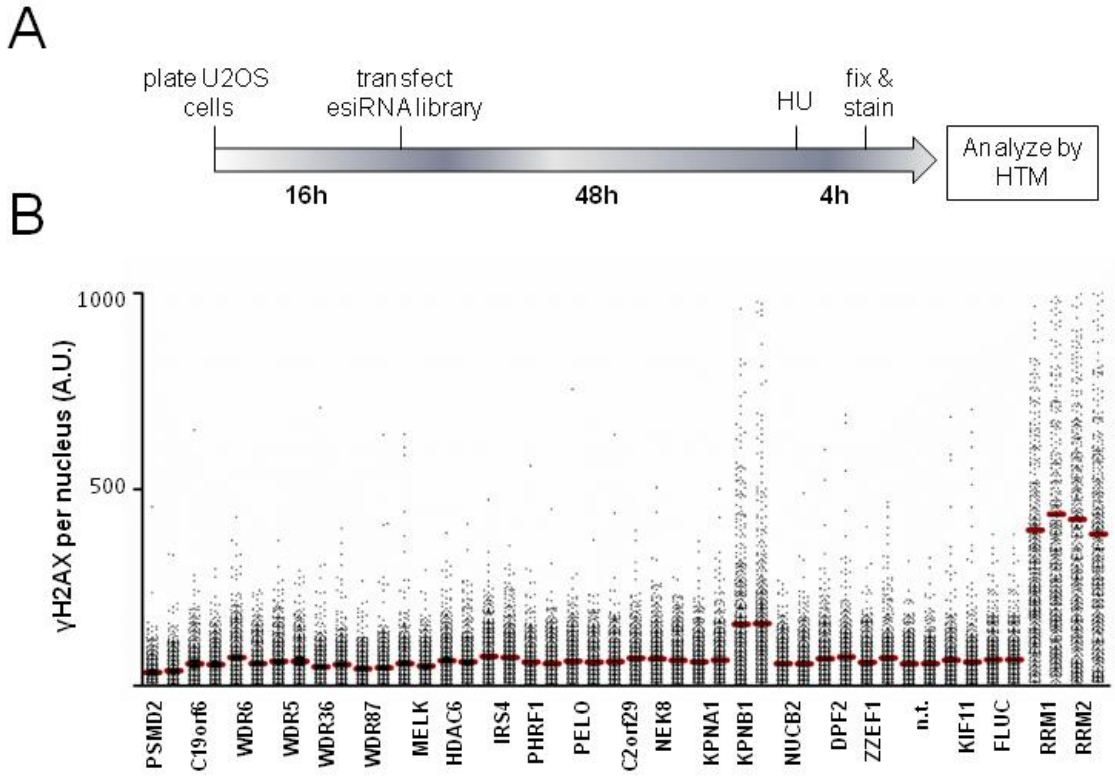


Figure 15: Screen for functional RNR-regulators. (A) Candidate genes were targeted by esiRNA transfection of U2OS cells. 48 hours after transfection, cells were treated with low doses of the RNR inhibitor HU, stained for γH2AX, and analyzed using the Opera HTM platform (B). A.U. = Arbitrary Units, n.t. = non transfected, FLUC = firefly luciferase (control). Data are representative of 3 independent experiments.

KPNB1, or importin-beta 1, is a nuclear importer that belongs to the importin-β family which consists of about 20 members in human. KPNB1 binds either directly or through karyopherin-alpha1 (KPNA1) to the nuclear localization signal (NLS) of its substrates, which are consequently shuttled into the nucleus via a RAN-GTP dependent mechanism. KPNB1 regulates a number of cellular functions such as cell cycle, mitosis and replication through the nuclear import of its substrates (Mosammaparast and Pemberton, 2004). Our results suggest a novel function for

KPNB1 in the regulation of the RS-response and a potential role in regulating RNR activity.

3.2 KPNB1 suppresses RS

In order to confirm our results for KPNB1 obtained in the genetic screening, we repeated the experiment above with 3 independent siRNAs targeting KPNB1. Depletion of KPNB1 by any of the three siRNAs increased the levels of γH2AX, which could be further increased by previous treatment with HU **(Figure 16)**.

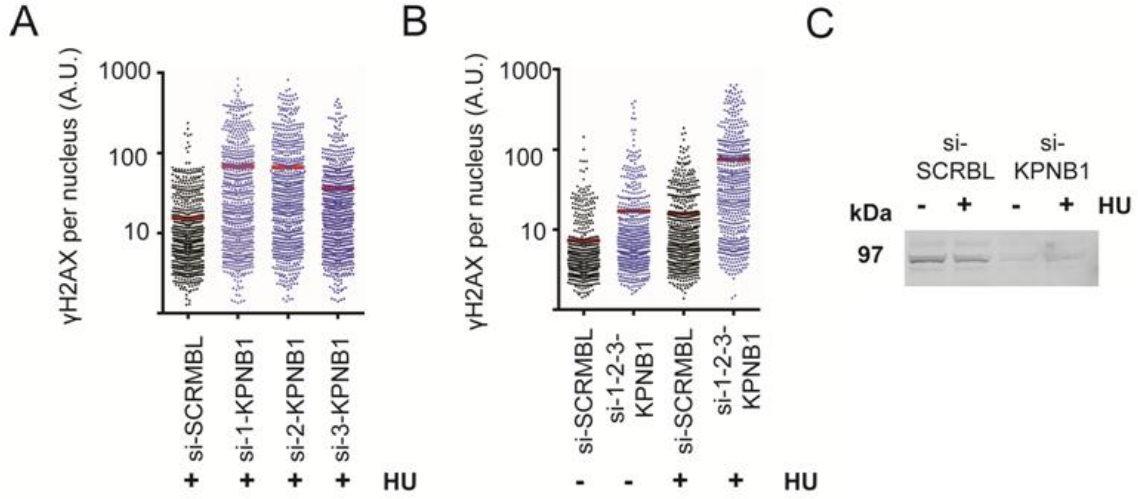


Figure 16: KPNB1 depletion induces RS. (A) HTM-mediated quantification of γH2AX intensity per nucleus in U2OS cells transfected with a scrambled control siRNA (si-SCRMBL) or one of three different siRNAs against KPNB1 (si-1-KPNB1, si-2-KPNB1 and si -3-KPNB1) and treated with HU for 4 hours. (B) HTM-mediated quantification of γH2AX intensity per nucleus in U2OS cells transfected with a scrambled control siRNA or a pool of the three siRNAs targeting KPNB1 (si-KPNB1-1,2,3) and treated with HU for 4 hours where indicated. Data from three independent experiments is shown. A.U. = Arbitrary Units. (C) WB of KPNB1 showing depletion in cells treated with a siRNA (si-1-KPNB1) against KPNB1.

To determine whether the role of KPNB1 on RS was direct, we tested whether its overexpression could increase the resistance towards RS. We generated a stable U2OS cell line overexpressing either KPNB1 or an empty control vector **(Figure 17A)**. Importantly, whereas KPNB1 expression did not affect DNA replication, as measured by EdU incorporation **(Figure 17B)**, it significantly reduced HU-induced

γH2AX levels **(Figure 17C)**. In summary, our results illustrate that KPNB1 suppresses HU-induced RS in human cells.

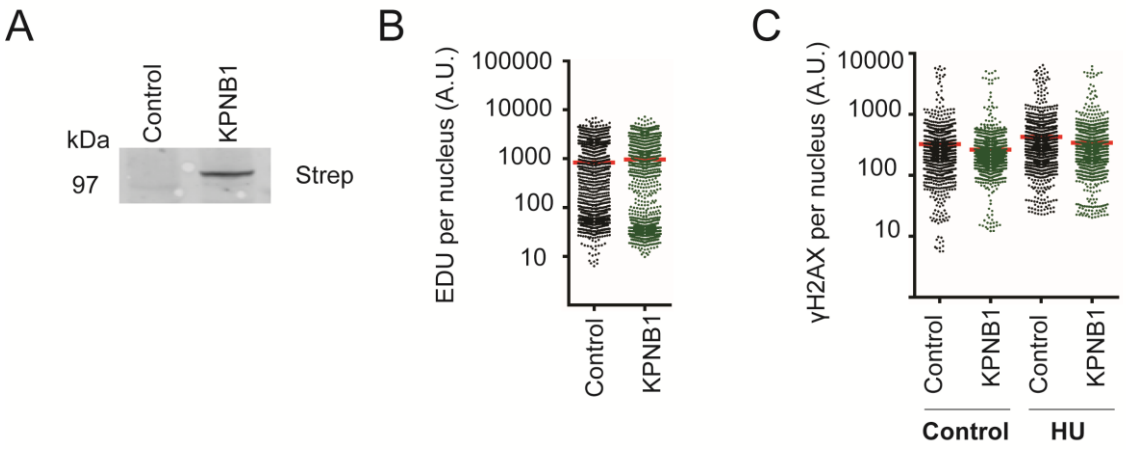


Figure 17: KPNB1 overexpression reduces RS. U2OS cells were infected with a lentivirus for KPNB1 or an empty vector (Control) and expression was validated by WB (A). (B) HTM-mediated quantification of EdU intensity per nucleus in control cells and cells overexpressing KPNB1. (C) Cells overexpressing either KPNB1 or empty vector were treated for 4 hours with 1mM HU and RS levels were quantified by HTM-mediated quantification of γH2AX intensity per nucleus. The experiment was done in duplicate and represents data of three independent experiments.

3.3 How does KPNB1 reduce RS?

Given that KPNB1 is a nuclear importer and that it was initially identified as a protein co-precipitating with RNR, we speculated that KPNB1 might facilitate the nuclear import of the RNR subunit(s) as a way to confer resistance towards RS. Moreover, studies in yeast that were published during this PhD identified a role for the yeast ortholog of KPNB1 in the translocation of RNR (Dosil, 2011). However, no difference in the distribution of RNR could be detected between control and KPNB1 deficient cells **(Figure 18)**. Interestingly, and by reasons that remain to be determined, KPNB1 depletion reduced the expression of RRM2 and RRM2B.

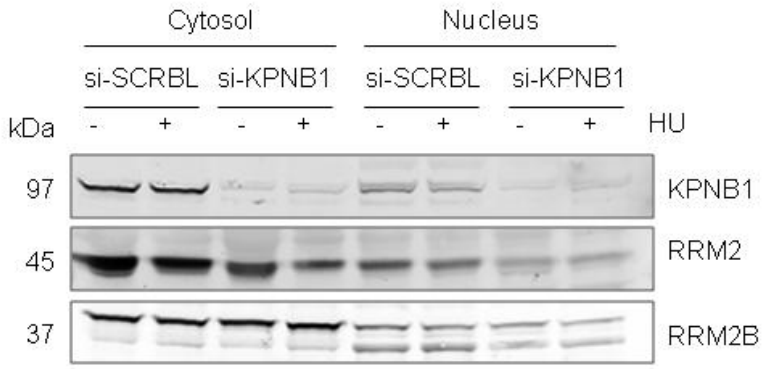


Figure 18: KPNB1 levels do not affect nuclear localization of RRM2. U2OS cells were transfected with a scrambled control siRNA (SCRBL) or siRNA targeting KPNB1, and after 48 hours cytosolic and nuclear fractions were analyzed by WB for RRM2 and RRM2B levels. Where indicated cells were treated with 2 mM HU for 4 hours.

Here we tried to identify new regulators of the mammalian RNR. In conclusion, our results suggest that the RNR is a rather stable complex composed of the RRM1, RRM2 and RRM2B subunits which do not seem to interact with other specific proteins at equimolar ratios. This study has also led to the identification of KPNB1 as a suppressor of HU-induced RS, although the mechanism remains to be defined.

4 A mouse model for the ribonucleotide reductase subunit 1

So far, we could show that indeed, the ATR–dependent regulation of RNR activity seems to be conserved in mammals to a large extent. However, the functional connection that links ATR to RNR remained elusive. As we have detailed before, an important factor that mediates the Mec1-dependent control over RNR in *S.cerevisiae* is its allosteric inhibitor Sml1. While no mammalian ortholog for Sml1 is known, the sequence surrounding the binding site of Sml1 to Rnr1 is well conserved and recombinant yeast Sml1 has been shown to bind and inhibit mammalian RRM1 *in vitro* (Chabes et al., 1999; Zhao et al., 2001). In 2000, Rothstein and colleagues described a point mutation in the *rnr1* gene (*rnr1-W688G*) in *S.cerevisiae* that leads to increased constitutive binding to Sml1 (Georgieva et al., 2000). The W688G mutation was originally identified based on its ability to physically interact with Sml1 mutants with compromised binding to wt Rnr1 (Georgieva et al 2000). The point mutation locates to a small alpha-helical region on the outside surface of the Rnr1 protein **(Figure 19A)** (Uhlin and Eklund, 1994), a region involved in the interaction of the Rnr1-NTD with Sml1 and the Rnr1-CTD (Zhang et al., 2007). Mutation of the Trp-688 to glycine caused a specific increase in Sml1 binding to Rnr1, resulting in reduced RNR activity, dNTP levels and cell viability; all of which could be rescued by concurrent deletion of Sml1 (Andreson et al., 2010; Georgieva et al., 2000). Interestingly, the sequence surrounding the point mutation is highly conserved from yeast to humans **(Figure 19B)**. We hypothesized that if a mammalian ortholog of Sml1 were to exist, introducing this mutation into the mouse genome might lead to increased binding of this factor to RRM1. In addition, such a mouse model would have constitutively lower levels of nucleotides, providing an interesting model to explore the role of nucleotide pools in mammalian health. We were particularly interested in testing whether *Rrm1-W684G* mice (from now on *Rrm1-WG*) would present a phenotype similar to yeast *rnr1-W688G*, such as reduced RNR activity and dNTP levels. Moreover, no loss-of-function mouse model of the RNR existed. Hence, we generated a mouse knock-in (KI) strain carrying the W684G mutation in *Rrm1*.

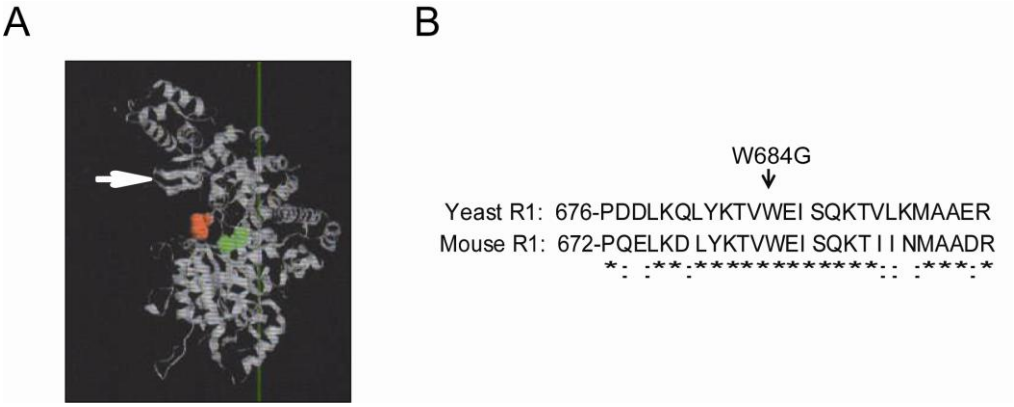


Figure 19: The WG point mutation is evolutionary conserved. (A) 3-D structure of *E.coli* R1. The position of the conserved W670 corresponding to W688 in yeast and W684 in mouse is indicated with a white arrow. The active site is shown in green and the two redox active cysteines are shown in red (Georgieva et al., 2000). (B) Pairwise local alignments of yeast (*Saccharomycis cerevisiae*) and mouse (*Mus musculus*) RRM1 protein sequences surrounding the point mutation.

4.1 Generation of the Rrm1^{WG} mouse model

Gene targeting into a desired genomic location allows for the genetic modification of a specific target sequence. We decided to use this method to generate a KI mouse model carrying the *Rrm1^{W684G}* point mutation. The mouse *Rrm1* gene is located to chromosome 7, has a total length of 28 kb and consist of 19 exons with W684 locating to exon 18. We designed a 16,8 kb construct containing exons 14-19 carrying the *Rrm1^{W684G}* mutation (TGG → GGC) and including downstream sequences of the *Rrm1* gene (chr7:102,460,313-102,481,519) **(Figure 20)**.

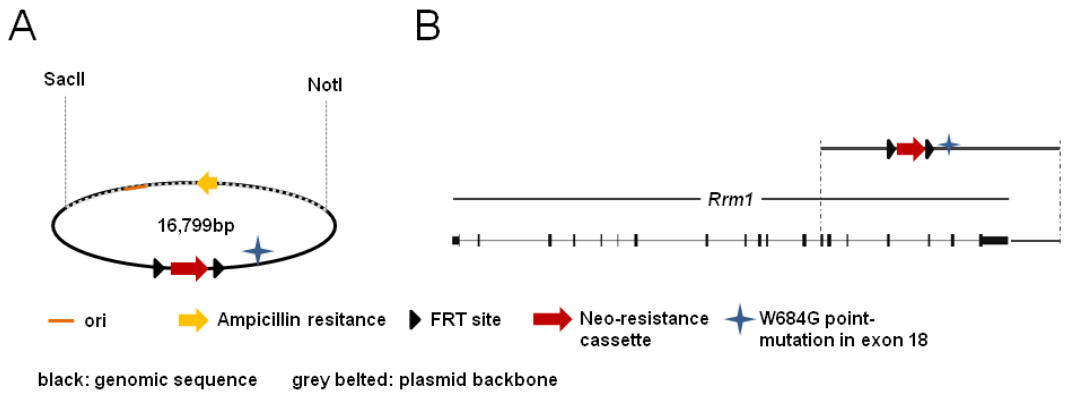


Figure 20: KI construct RRM1. (A) *Rrm1* knock-in construct comprising a FRT-neo-FRT resistance cassette and exon 18 harbouring the W684G point mutation flanked by two 6kb homology arms. The vector was linearized with SacII and NotI and electroporated into ES cells. (B) Genomic integration site within *Rrm1* and location of the W684G point mutation.

The construct was generated by recombineering (GeneBridges) and the linearized *Rrm1-WG* targeting vector was electroporated into mouse embryonic stem (ES) cells by the Transgenic Mice Unit of the CNIO. The presence of a Frt-Neo-Frt resistance cassette allowed to select successfully transfected ES cells with Neomycin resistance. ES cell lines carrying the *Rrm1-WG* mutation were identified by southern blotting and one line ($Rrm1^{WG(Neo)}$) carrying the correct integration was selected **(Figure 21)**.

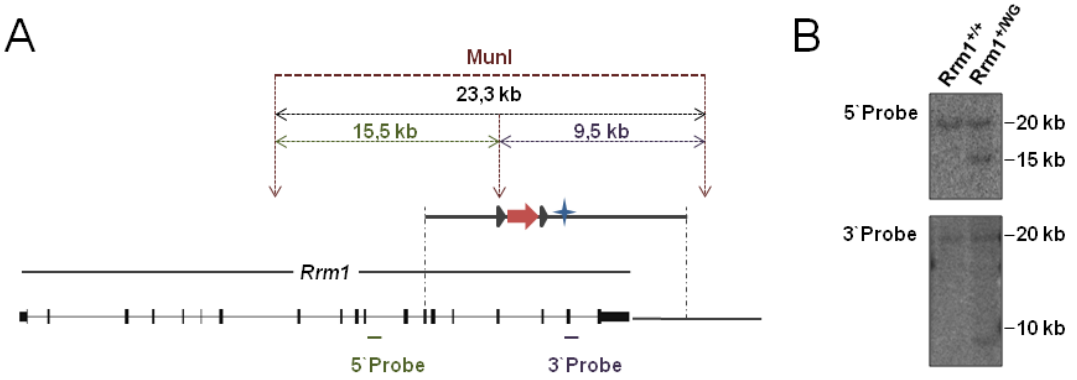


Figure 21: Strategy and identification of $Rrm1^{+/WG(Neo)}$ ES cells. (A) Schematic figure of Southern blot strategy indicating the restriction sites for MunI and the binding sites of the radioactively labeled probes. (B) Southern blot with an external (5´Probe) and internal (3´Probe) *Rrm1* probe, illustrating the presence of an integration site (15,5 kb and 9,5 kb respectively) on the $Rrm1^{WG\ (Neo)}$ strain. The 23,3 kb band corresponds to the endogenous *Rrm1*.

The *Rrm1*$^{WG(Neo)}$ ES cell line was subsequently used for the aggregation with blastocyst stage embryos followed by implantation into a pseudo pregnant female and a chimeric *Rrm1*$^{WG(Neo)}$ founder line was established. *Rrm1*$^{WG(Neo)}$ KI lines were identified by PCR using primers annealing to the sequence coding for the neomycin resistance gene within the targeting vector. To remove the Neo-resistance cassette from the genome, *Rrm1*$^{WG(Neo)}$ mice were crossed with CAG-Flpe transgenic mice that express the enhanced version of the FLP recombinase under the control of the synthetic CAG promoter providing ubiquitous expression in all tissues (Rodriguez et al., 2000). FLP recombinase detects the FRT-FRT sites flanking the resistance cassette and mediates recombination that will result in deletion of the FRT-flanked Neomycin resistance gene in the offspring **(Figure 22)**.

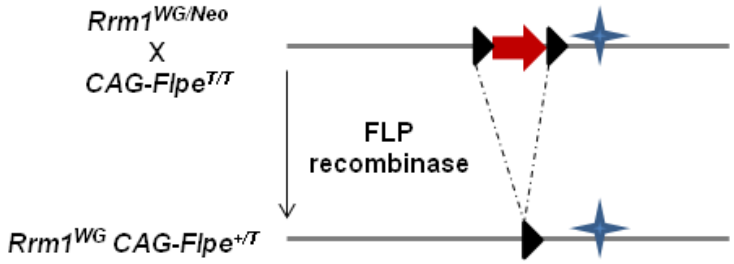


Figure 22: FLP recombinase mediated removal of the Neomycin cassette. Crossing of *Rrm1*$^{WG(Neo)}$ mice with *CAG-Flpe*$^{T/T}$ animals leads to FLP-recombinase dependent removal of the Neo-resistance cassette (red arrow) in the offspring using the FRT sites (black triangle).

Accordingly, *Rrm1*$^{WG(Neo)}$ mice were bred with *CAG-Flpe*$^{T/T}$ mice in order to remove the resistance cassette. Removal of the Neomycin cassette was confirmed by PCR and a new Neo-cassette free line, *Rrm1*^{WG}, was established. All subsequent experiments described in the thesis were done with this line, which only carries the *Rrm1-W684G* mutation and an FRT in its genome.

4.2 The Rrm1-W684G mutation causes early embryonic lethality in mice

The yeast *rnr1-WG* point mutation causes increased binding of Sml1 to Rnr1 resulting in reduced cellular fitness, RNR activity and dNTP levels, while deletion of Sml1 rescued those phenotypes (Andreson et al., 2010; Georgieva et al., 2000). In order to study the consequences of $Rrm1^{WG}$ on mammalian health, we aimed to generate mice carrying the $Rrm1^{WG}$ mutation in homozygosity. However, when intercrossing heterozygous $Rrm1^{+/WG}$ mice we observed that the $Rrm1^{WG/WG}$ point mutation led to homozygous lethality. No homozygous pups were born, nor were we able to detect homozygous mutant embryos at earlier developmental stages, demonstrating that $Rrm1^{WG/WG}$ causes early embryonic lethality in mice **(Figure 23)**.

A

	$Rrm1^{+/+}$	$Rrm1^{+/WG}$	$Rrm1^{WG/WG}$	(n)
Adult	39	61	0	80
13,5 dpc	35	64	0	62
3,5 dpc	28	71	0	7

B

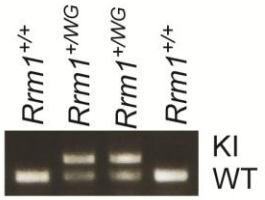


Figure 23: $Rrm1^{WG/WG}$ is homozygous lethal. (A) Table showing the observed genotypes of 3 week old pups, 13.5 dpc or 3.5 dpc embryos, obtained from crosses of $Rrm1^{+/WG}$. (B) Genotyping PCR illustrating the two *Rrm1* genotypes obtained.

In mammals, the RRM1 subunit is believed to be present in excess while the RRM2 subunit is rate limiting for enzyme activity (Engstrom et al., 1985). Hence, we explored whether the lethality of $Rrm1^{WG/WG}$ mice could be rescued by increased levels of RRM2. To this end, we crossed $Rrm1^{+/WG}$ heterozygous animals with the $Rrm2^{Tg}$ strain. However, increased RRM2 levels did not rescue homozygous lethality of the WG mutation as we failed to detect animals homozygous for $Rrm1^{WG}$ even in a genetic background of $Rrm2^{Tg}$ **(Table 2)**. Collectively, these results reveal that the W684G mutation is essential for mammalian viability, in a way that cannot be rescued by an additional supply of RRM2.

	$Rrm1^{+/+}$	$Rrm1^{+/WG}$	$Rrm1^{WG/WG}$
$Rrm2^{+/+}$	5	8	0
$Rrm2^{+/T}$	14	19	0
$Rrm2^{T/T}$	13	12	0

Table 2: Supraphysiological levels of RRM2 do not rescue *Rrm1*$^{WG/WG}$ lethality. Table showing the observed genotypes of 3 week old pups, obtained from intercrossing $Rrm1^{+/WG}$ $Rrm2^{Tg}$ animals.

4.3 Rrm1$^{+/WG}$ *mice are phenotypically normal*

The early homozygous lethality of the RRM1-WG point mutation indicated that RNR function was severely compromised in $Rrm1^{WG/WG}$ mice, what prevented further characterization of the physiological consequences of $Rrm1^{WG}$ in mammals. Observing such a drastic effect of $Rrm1^{WG/WG}$ on the murine health, we analyzed the physiological consequences in mice carrying the $Rrm1^{WG}$ point mutation in heterozygosity. In contrast to $Rrm1^{WG/WG}$, $Rrm1^{+/WG}$ mice were fertile, born at mendelian ratios and showed no obvious phenotype **(Figure 23 and 24)**. The size and body weight of $Rrm1^{+/WG}$ mice were normal, indicating that the animals did not suffer from an overall deficiency in growth or proliferation rate **(Figure 24)**.

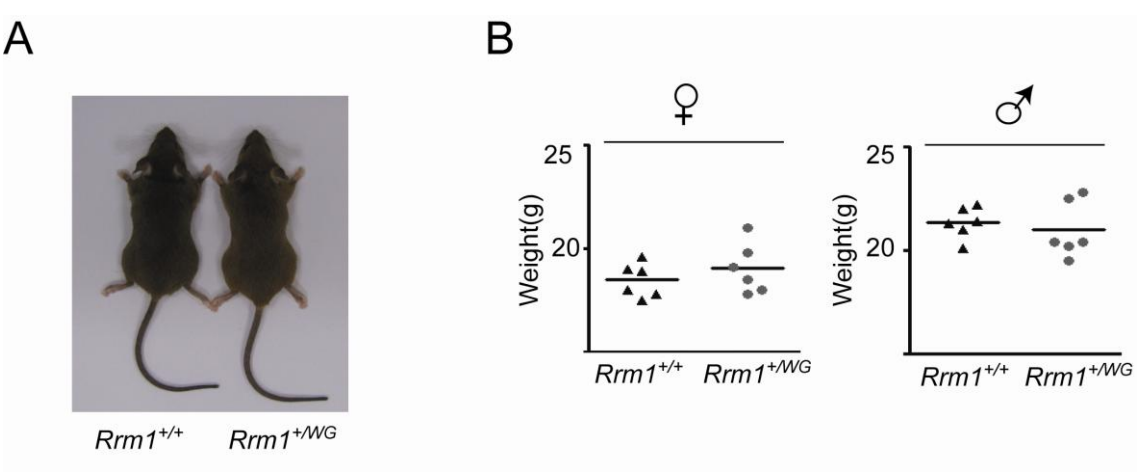


Figure 24: *Rrm1*$^{+/WG}$ mice are phenotypically normal. A) Representative picture of 4 month old wt and $Rrm1^{+/WG}$ littermates. (B) Weight distribution of 2 month old wt and $Rrm1^{+/WG}$ mice.

To determine if the essential nature of the Rrm1-WG mutation was due to an effect on protein levels, we first verified whether $Rrm1^{+/WG}$ cells presented normal expression of the mutant allele. Indeed, RT-PCR followed by sequencing revealed equivalent expression of the mutant and wt mRNAs in $Rrm1^{+/WG}$ cells **(Figure**

25A,B). Western blotting also failed to show any noticeable differences in RRM1 protein levels between wt and heterozygous cells **(Figure 25C)**. Hence, wt and RRM1-WG versions of RRM1 coexist at similar levels in *Rrm1*$^{+/WG}$ cells.

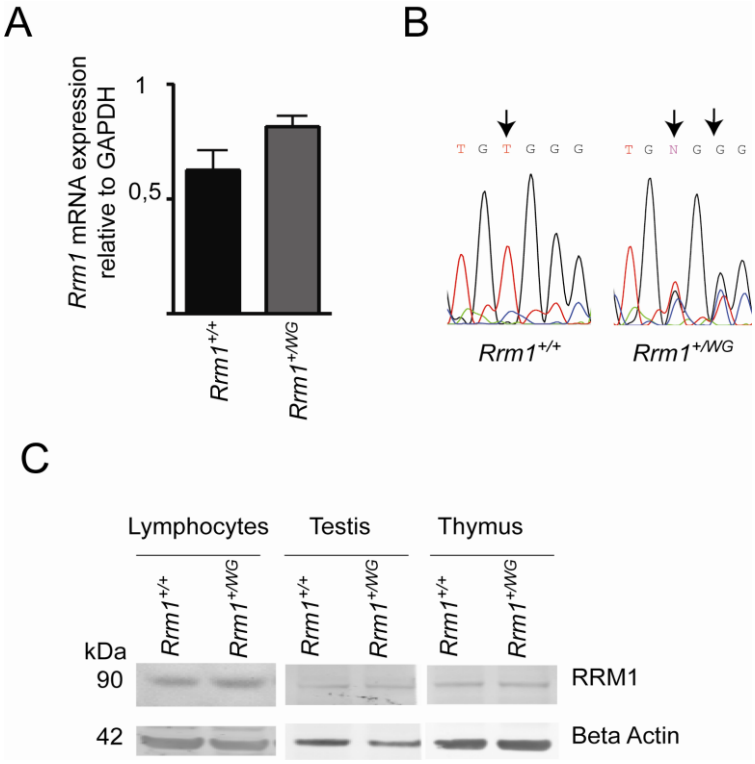


Figure 25: *Rrm1-WG* is normally expressed and *Rrm1*$^{+/WG}$ cells exhibit normal RRM1 protein levels. (A) Real-time PCR (RT-PCR) analysis of *Rrm1* expression levels in wt or *Rrm1*$^{+/WG}$ MEF. Results are representative of three independent experiments performed in triplicate. (B) Sequence chromatograms showing the mutations (TGG →GGC) generated in *Rrm1-WG* mice. The arrows indicate the positions of the mutations. (C) RRM1 Western blot analysis of B lymphocytes, testis and thymus from wt and *Rrm1*$^{+/WG}$ mice. Beta Actin was used as a loading control.

4.4 RRM1-WG overexpression does not induce RS

To overcome the limitations imposed by the lethality of the mutation in mice and further explore the consequences of RRM1-WG expression in mammals, we generated constructs comprising human RRM1 or RRM1-WG with a C-terminal Strep-tag that were stably expressed in the doxycycline-inducible FlpIn T-REx 293 cell line **(see Figure 13).** The RRM1-(WG-)-Strep constructs were then used to

generate a doxycycline-inducible FlpIn T-Rex 293 cell line. First, we observed that the subcellular distribution and stability of RRM1-WG were comparable to those of wt RRM1 **(Figure 26A)**.

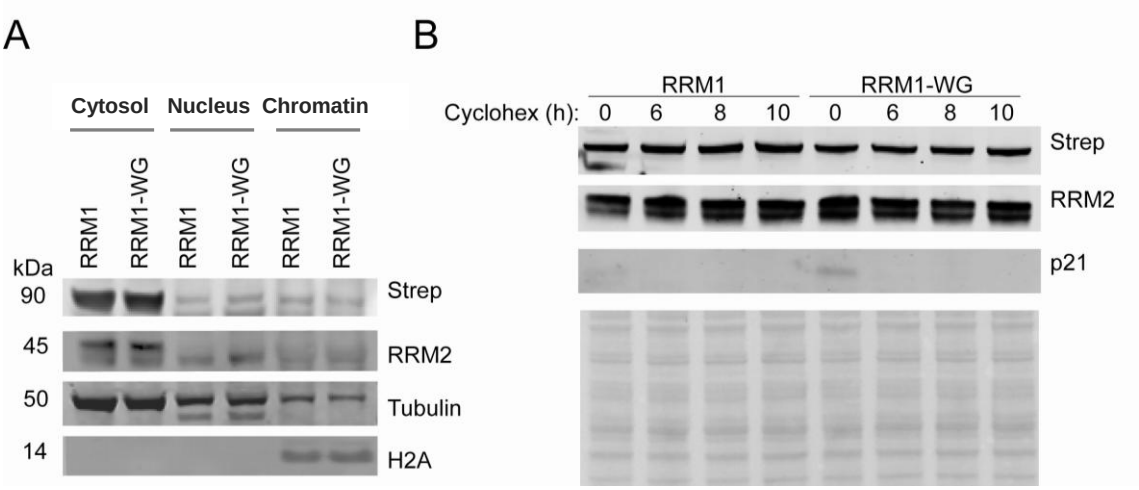


Figure 26: RRM1-WG exhibits normal cellular distribution and protein stability. (A) WB analysis of cytoplasmic, nuclear soluble and nuclear pellet fractions of FlpIn T-REx 293 cells overexpressing RRM1-Strep or RRM1-WG-Strep. Tubulin and H2A were used as controls for cytosolic and chromatin fractions respectively. (B) WB analysis of FlpIn T-REx 293 cells overexpressing RRM1-Strep or RRM1-WG-Strep after incubation with 25 μg/ml cycloheximide for the indicated time points. p21 was used as control for protein degradation.

Moreover, and in agreement with the absence of obvious phenotypes on *Rrm1*$^{+/WG}$ mice, overexpression of RRM1-WG did not affect cell cycle progression nor had it any obvious impact on RNR activity as measured by HU-induced H2AX phosphorylation **(Figure 27).** Altogether, these results indicate that lethality of *Rrm1*$^{WG/WG}$ mice is not linked to lower levels, altered distribution or intrinsic toxicity of the RRM1-WG protein.

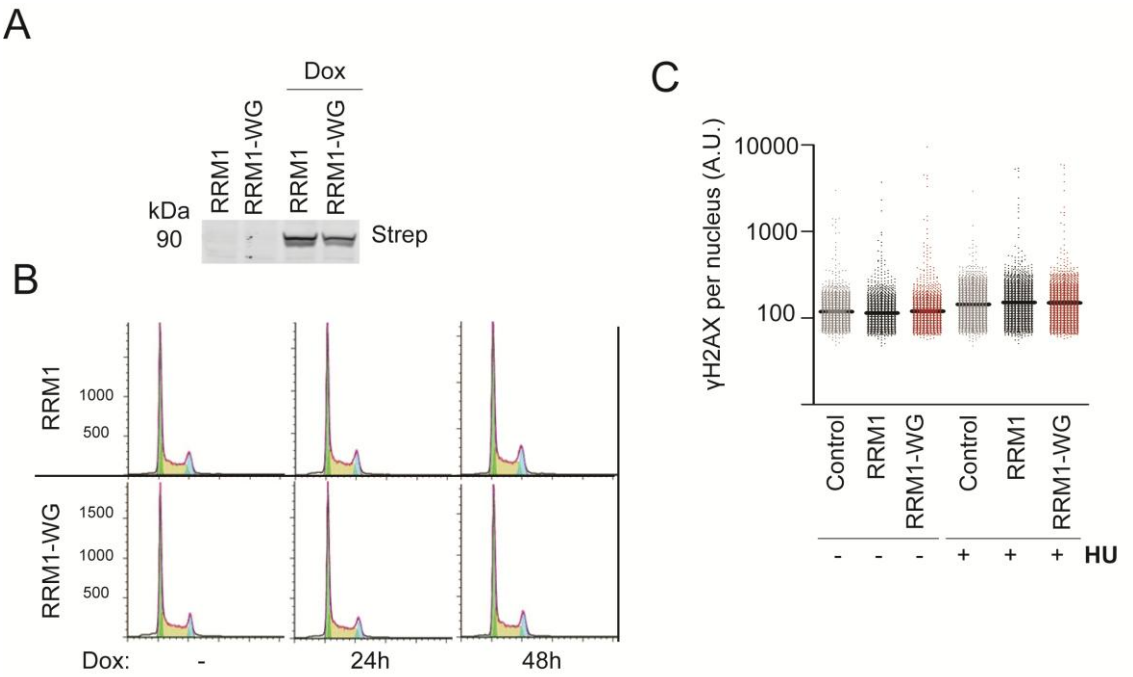


Figure 27: Overexpression of RRM1-WG does not affect the cell cycle or RS. (A) WB and (B) cell cycle-analysis of Flp-In T-REx 293 cells expressing RRM1-Strep or RRM1-WG-Strep after 24 (and 48) hours of doxycycline (Dox) induction. (C) HTM-mediated quantification of γH2AX intensities in Flp-In 293 T-REx cells expressing empty Strep vector (Control), RRM1-Strep or RRM1-WG-Strep treated with 0,5 mM HU for 4 hours. Data are representative of three independent analyses. A.U.= Arbitrary Units.

4.5 Rrm1^{WG} heterozygosity does not impact the response to DNA damage in cells and mice

We first obtained MEFs from *Rrm1$^{+/WG}$* mice and wt littermates both of which exhibited normal growth **(Figure 28)**.

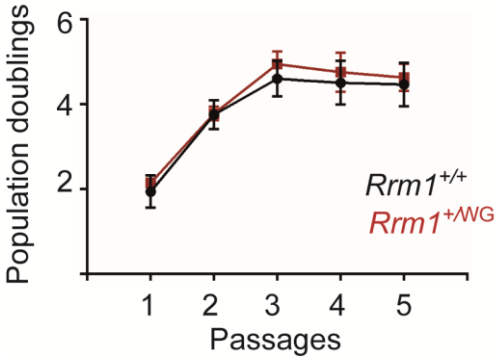


Figure 28: *Rrm1$^{+/WG}$* MEF proliferate normally. Growth curve of pairs of three *Rrm1$^{+/+}$* and *Rrm1$^{+/WG}$* MEF lines derived from littermates. Data are representative of two independent analyses.

Despite the overall normal appearance of *Rrm1*$^{+/WG}$ mice, we explored whether the presence of the mutation might be more relevant in conditions that demand an extra supply of dNTPs, such as during DNA repair. To investigate whether RNR function was even slightly compromised in *Rrm1*$^{+/WG}$, we exposed *Rrm1*$^{+/+}$ and *Rrm1*$^{+/WG}$ MEFs to HU and compared the levels of γH2AX. HTM analyses revealed that HU-induced γH2AX levels were not altered on *Rrm1*$^{+/WG}$ MEFs when compared to those observed on littermate MEFs **(Figure 29)**.

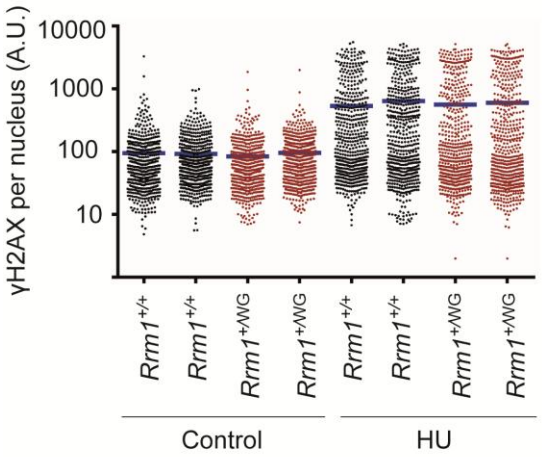


Figure 29: MEF from *Rrm1*$^{+/WG}$ exhibit a normal RS-response. HTM-mediated quantification of γH2AX intensities in *wt* and *Rrm1*$^{+/WG}$ MEF treated with 0,5mM HU for 4 hours. Data are representative of three independent analyses. A.U.= Arbitrary Units

Given that MEFs are a slow proliferating cell type, they are not particularly prone to RS. In contrast, lymphocytes undergo rapid proliferation cycles and therefore might have a more important demand of nucleotides. We purified B-cells from *Rrm1*$^{+/WG}$ and *Rrm1*$^{+/+}$ mice and challenged them with HU or ionizing radiation (IR). Consistent with data from MEFs, phosphorylation of CHK1 and RPA were not affected in *Rrm1*$^{+/WG}$ B-cells **(Figure 30).**

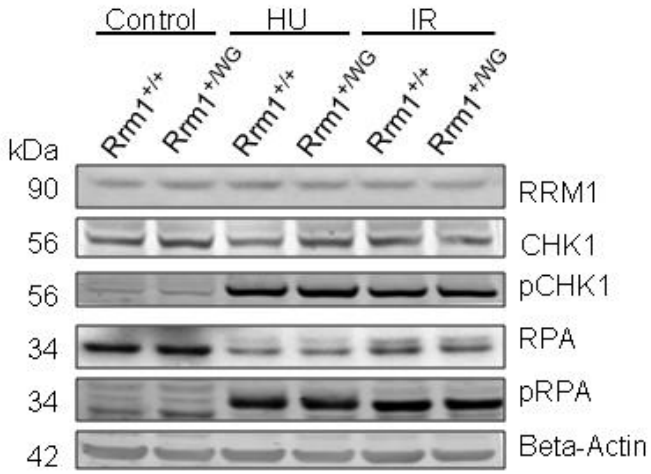


Figure 30: Lymphocytes isolated from *Rrm1$^{+/WG}$* exhibit a normal induction of the RS-response. RRM1, phospho CHK1, CHK1, phospho RPA, and RPA Western blot in wt and *Rrm1$^{+/WG}$* littermate B-cells, either untreated (Control) or upon treatment with 2mM HU for 3 hours or 10Gy IR. Data are representative of two independent analyses. β-actin was used as a loading control.

While we failed to detect an increase in the sensitivity towards RS or DNA damage inducing agents in *Rrm1$^{+/WG}$* cells *in vitro*, we speculated that *Rrm1$^{+/WG}$* animals could show more phenotypes when exposed to RS inducing conditions. Given that the hematopoietic stem cell (HSC) compartment is particularly sensitive to RS and DNA damage (Flach et al., 2014; Murga et al., 2009; Rossi et al., 2007) we evaluated the behavior of *Rrm1$^{+/WG}$* HSC *in vivo*. To this end, we tested how wt and *Rrm1$^{+/WG}$* HSC repopulate the bone marrow of mice exposed to a sub-lethal dose of IR. In agreement with *in vitro* data, hematopoietic recovery of *Rrm1$^{+/WG}$* and *Rrm1$^{+/+}$* mice showed no significant differences **(Figure 31)**. Collectively, the results above show that the expression of RRM1-WG to around half of the total amount of RRM1 does not have a detectable impact on mammalian cells, supporting the notion that RRM1 exists largely in excess and that RNR activity is mostly dependent on RRM2.

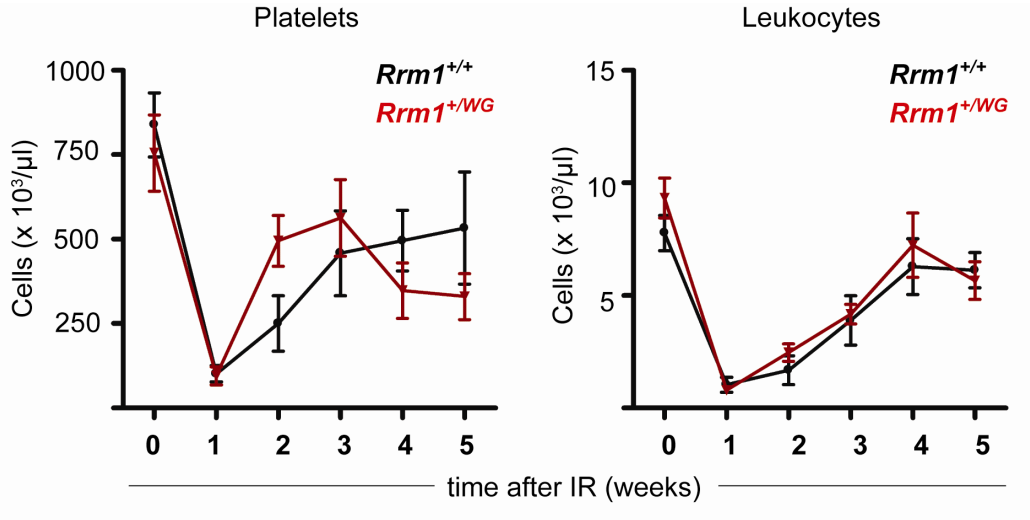
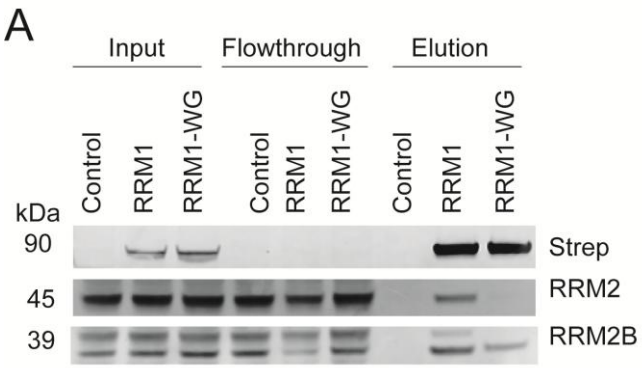


Figure 31: *Rrm1*$^{+/WG}$ mice show a normal IR recovery. Hematopoietic recovery after a sublethal dose of irradiation (6 Gray). Blood indices from *Rrm1*$^{+/WG}$ mice are compared with wt mice at 1-5 weeks post irradiation. Values are means ± SD, n = 6.

4.6 RRM1-WG does not form the RNR holocomplex

Even if *Rrm1*$^{+/WG}$ mice and cells had no obvious phenotype, the lethality of the allele in homozygosis indicated that the mutant protein was not functional. In yeast, the Rnr1-WG mutant leads to constitutive binding of the RNR inhibitor Sml1 to Rnr1. In theory, the binding of murine RRM1-WG to a yet-unidentified RNR inhibitor could also explain the lethality of *Rrm1*$^{WG/WG}$ mice. To search for proteins that more avidly bind to RRM1-WG than to wt RRM1, we purified Strep-tagged RRM1 proteins and, with the help of the Proteomics Unit of the CNIO, looked for interactors by MS. However, no proteins specifically enriched in the RRM1-WG pull-downs were identified. In contrast, we observed a major reduction in the number of RRM2 and RRM2B peptides identified in RRM1-WG pull-downs **(Figure 32A)**. WB analysis confirmed a very significant reduction of the levels of RRM2 and RRM2B in RRM1-WG pull-downs **(Figure 32B)**. The impaired formation of the RNR complex explains both the lethality of *Rrm1*$^{WG/WG}$ mice and the absence of toxic effects of the mutant protein even when overexpressed.



B

Protein	PSM Control	PSM RRM1	PSM RRM1-WG
RRM1	11	1569	946
RRM2	0	58	8
RRM2B	1	49	4

Figure 32: RRM1-WG interferes with RNR heterocomplex formation. (A) Strep-IP from total cell lysate of Flp-In T-REx 293 cells overexpressing an empty control vector, RRM1-Strep or RRM1-WG-Strep protein. (B) Protein spectrum matches (PSM) of RRM2 and RRM2B identified by mass spectrometry in the fraction containing purified RRM1 or RRM1-WG.

4.7 RRM1-WG binds RRM2(B) in vitro

While the loss of RNR complex formation could explain the lethality observed in *Rrm1*$^{WG/WG}$ mice, this finding stands in contrast to the impact of the mutation in *S.cerevisiae*. Whereas the *rnr1-WG* mutation caused increased Rnr1-Sml1 binding, the formation of Rnr1-Rnr2 complexes was not compromised. Moreover, deletion of Sml1 rescued the Rnr1-WG phenotypes (Andreson et al., 2010). Interestingly, Chabes and colleagues reported that recombinant yeast Sml1 competes with RRM2 binding to human RRM1, disrupting RNR complexes (Chabes et al., 1999). Hence, our observations of lower levels of RRM2 in RRM1-WG-Strep pull-downs could be explained in two different ways. The W684G mutation could directly impair the binding of murine RRM1 to RRM2. Alternatively, the mutation could increase the binding of RRM1 to another protein, precluding the RRM1-RRM2 interaction. In order to distinguish between those two possibilities, we analyzed the *in vitro* capacity of RRM1-WG to form the RNR complex**.** We expressed and purified mutant and wt RRM1, as well as RRM2 and RRM2B proteins with an N-terminal His-tag from *Escherichia coli.* Additionally, RRM1 proteins were purified with an N-terminal Strep-tag to allow for an independent purification **(Figure 33 and 34)**.

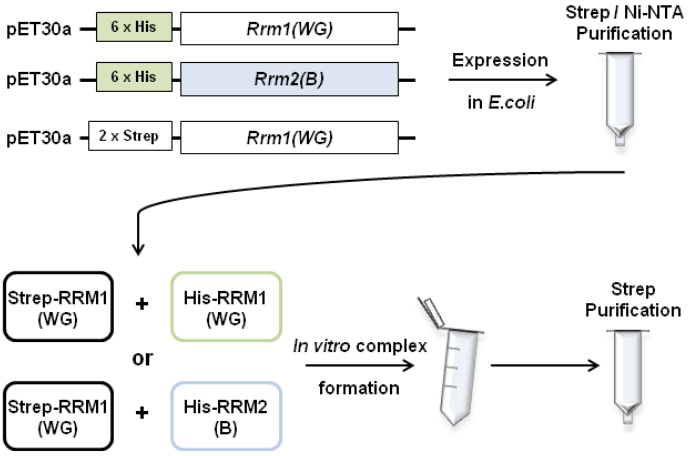


Figure 33: Experimental procedure to test the RRM1-WG RNR complex formation in vitro. Human *Rrm1(WG), Rrm2 and Rrm2B* with an N-terminal His tag were expressed in *E.coli* cells and purified with Ni-NTA beads. Similarly, human *Rrm1(WG)* with an N-terminal Strep tag was expressed in *E.coli* and purified with Strep-tactin beads. Purified Strep-RRM1 or Strep-RRM1-WG protein was then incubated with His-RRM1(WG) or His-RRM2(B) and purified by Strep-IP.

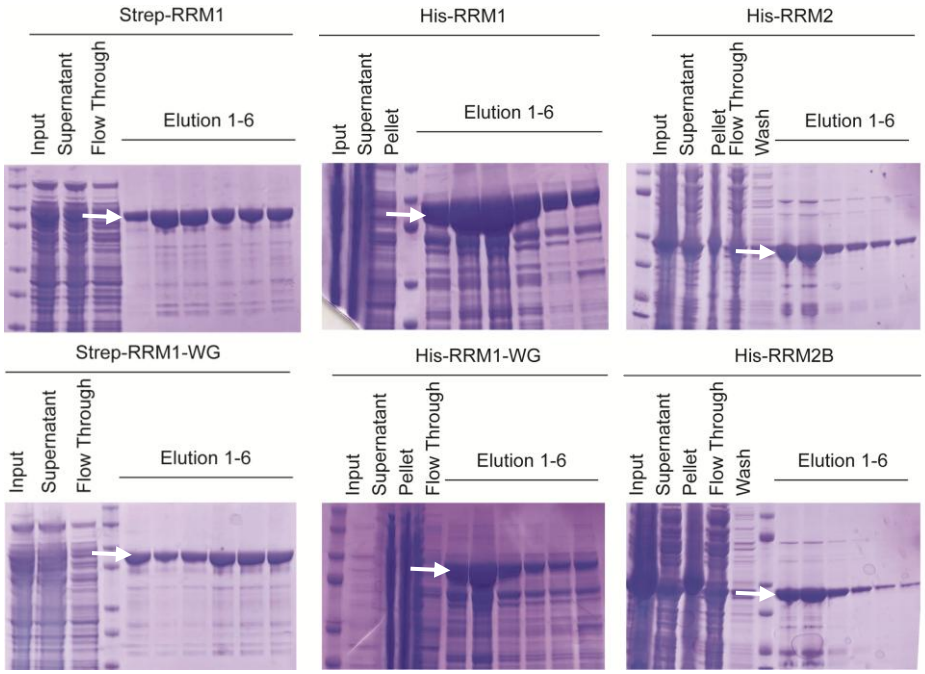


Figure 34: Coomassie staining of the purification of Strep-RRM1, Strep-RRM1-WG, His-RRM1, His-RRM1-WG, His-RRM2 and His-RRM2B. The white arrow indicates the band representing the purified protein.

Using purified proteins, we first observed that RRM1-WG can bind to wt RRM1 and form the RRM1 homodimer **(Figure 35)**.

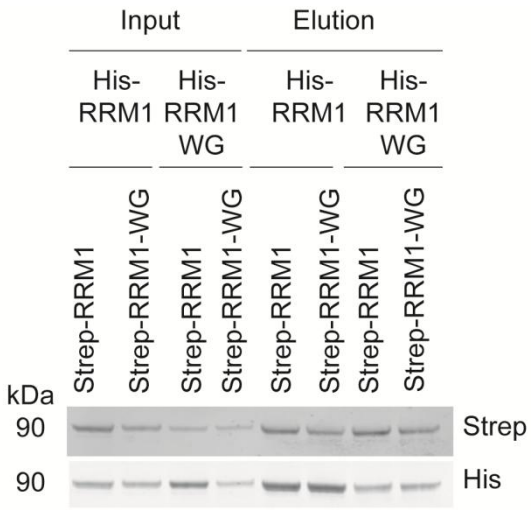


Figure 35: RRM1-WG forms the RRM1 homodimer. Strep and His WB analysis of Strep IP after incubation of Strep-RRM1 or Strep-RRM1-WG with His-RRM1 or His-RRM1-WG.

Next, we tested whether recombinant RRM1-WG was able to bind RRM2 or RRM2B *in vitro*. Indeed, recombinant RRM1-WG bound both RNR regulatory subunits as efficiently as wt RRM1 **(Figure 36)**. Because RNR holocomplex formation can be affected by binding of its effector nucleotides (Ahmad and Dealwis, 2013), we also tested complex formation in the presence and absence of physiological levels of ATP. Again, we observed no effect of the WG mutation on the binding to RRM2(B) (data not shown). Thus, the introduction of the W684G point mutation in murine RRM1 does not intrinsically affect its binding to RRM2 or RRM2B.

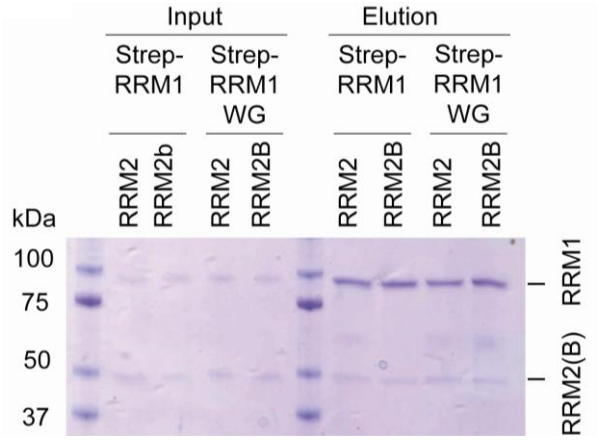
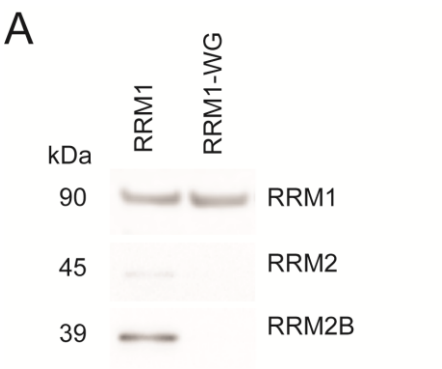


Figure 36: RRM1-WG binds RRM2(B) *in vitro*. Coomassie staining of Strep-IP after *in vitro* incubation of Strep-RRM1 or Strep-RRM1-WG with His-RRM2 or RRM2B.

4.8 RRM1-WG does not bind RRM2(B) in cells

The observation that RRM1-WG was proficient in forming the RNR complex *in vitro* indicated that another factor could interfere with RRM1-WG binding to RRM2 *in cellulo*. This factor might be a protein modification (e.g. phosphorylation of RRM1 or RRM2) or another feature of the cellular environment that was absent from our *in vitro* experimental setup. Additionally, and as suggested in yeast, it could also be that another protein competed with the small RNR subunits for binding to RRM1. To further search for factors that could bind preferentially to RRM1-WG, Strep-RRM1 and Strep-RRM1-WG proteins were reversibly linked to Strep-tactin beads and used as baits for pull-downs. Once again, MS analysis did not reveal any preferential interactors of RRM1-WG. In contrast, and in agreement with our previous data, recombinant RRM1-WG failed to pull-down RRM2 and RRM2B as efficiently as wt RRM1 **(Figure 37)**. In summary, whereas RRM1-WG can bind to RRM2(B) *in vitro* it fails to form a RNR complex *in vivo*, which explains the lethality of *Rrm1*$^{WG/WG}$ mice.



B

Protein	PSM RRM1	PSM RRM1-WG
RRM1	254	266
RRM2	3	1
RRM2B	9	0

Figure 37: RRM1-WG fails to bind RRM2(B) in cellular extracts. A) WB and B) Protein spectrum matches (PSM) of RRM2 and RRM2B protein identified by mass spectrometry in the fractions incubated with recombinant Strep-RRM1 or Strep-RRM1-WG protein reversibly linked to Strep-tactin beads.

Collectively, our results demonstrate that the Rrm1-WG mutation also compromises RNR function in mammals, but through a different mechanism to what has been described for yeast. In mice, the RRM1-WG mutant protein fails to interact with RRM2(B) leading to early embryonic lethality. Whereas RRM1-WG

can bind to RRM2 using recombinant proteins, it does not in cells, in a way that could be consistent with the presence of a yet to be identified factor that competes with the RRM1-RRM2 binding. In contrast to the lethal effect of the *Rrm1*^{WG} mutation in homozygosity, heterozygous mutant mice have no phenotype, supporting the idea that protein levels of RRM1 are not limiting for RNR function.

DISCUSSION

The balanced regulation of dNTP levels is crucial to sustain proliferation while preventing mutagenesis. Previous work in model organisms suggests that ATR, the main checkoint kinase, safeguards genomic integrity by directly stimulating dNTP production upon demand and it has been speculated that it regulates the dNTP pool. In fact, yeast ATR ortholog Mec1 acts as a critical regulator of the nucleotide pool by activating RNR. In mammals, a relationship between nucleotide supply and oncogene-induced RS has recently been established (Bester et al., 2011), together with a connection between ATR and RNR to provide dNTPs during DNA repair (D'Angiolella et al., 2012). However, a direct connection between ATR signaling and RNR activity in the context of normal and aberrant DNA replication in mammals had not been explored in depth. In this work, we tried to identify new regulators of RNR activity, aimed to explore whether the ATR-dependent regulation of the RNR is conserved in mammals and how this regulation is established.

1 ATR-dependent transcriptional regulation of the RNR is conserved in mammals

Several lines of evidence support a critical function of ATR in regulating the RNR in mammals. Best defined is the role of ATR in the regulation of transcription of the RNR subunits, although conflicting data exist **(see Introduction 2.2.2)**. Here, we observed that ATR not only induces the expression of *Rrm2*, but also increases the transcription of *Rrm1*. Importantly, artificially induced ATR signaling, in the absence of actual DNA damage, was sufficient to stimulate RNR transcription excluding the possibility that our results were biased by ATM signaling (Eaton et al., 2007). Interestingly, *Rrm2b* expression is not downregulated but rather overexpressed in response to CHK1 inhibitors, which could be explained by the fact that *Rrm2b* is a p53 target and CHK1 inhibition generates DNA breakages. Our

results support a conservation of the Mec1(ATR)-Rad53(CHK1)-dependent control of the expression of RNR genes in mammals. However, it remains unclear whether CHK1 induces *Rrm1* transcription directly or via downstream mediators. In yeast, Mec1-Rad53 signaling in response to replication blocks is mediated via Dun1 and the transcriptional repressor of *rnr2/rnr4/rnr3*, Crt1. The mammalian ortholog of Crt1 is RFX1, which regulates *Rrm2* expression in response to ATR signaling through a conserved mechanism (Lubelsky et al., 2005). Although RFX1 contains a consensus CHK1 phosphorylation site (O'Neill et al., 2002), it has not been determined whether RFX1 acts downstream of CHK1 or is being directly phosphorylated by ATR (Lubelsky et al., 2005). It has also been proposed that *Rrm2* is controlled via ATR/ATM-CHK1-E2F1 during S-phase (DeGregori et al., 1995) and in response to DNA damage (Zhang et al., 2009). Whether RFX1 or E2F1 also mediate the ATR-CHK1-dependent regulation of *Rrm1* expression remains to be adressed **(Figure 38)**.

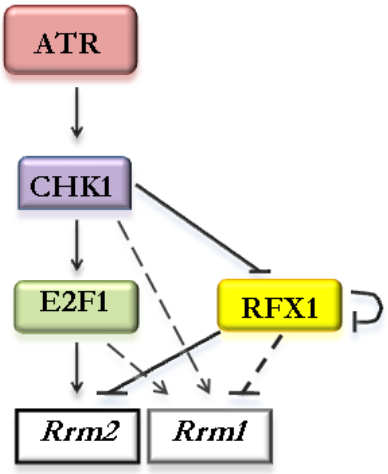


Figure 38: ATR-dependent transcriptional regulation of the canonical RNR subunits is conserved in mammals. ATR-CHK1 signaling induces E2F1, which in turn induces *Rrm2*. *Rrm2* expression is also induced in response to ATR-dependent phosphorylation of its inhibitor RFX1. Whether ATR-CHK1-dependent *Rrm1* expression is mediated via RFX1, E2F1 or another mechanism is currently not known.

2 Is there a role for nuclear RNR?

In yeast, Mec1 regulates RNR at different levels, from its subcellular localization and activity to its transcription. Although traditionally dNTP synthesis is believed to be a cytosolic process, it has been a matter of speculation whether part of the nucleotide metabolism is conducted within the nucleus. Although all mammalian RNR subunits are mainly cytosolic, the need for compartmentalization of nucleotide metabolism to the RF has been proposed based on the following observations: 1) intracellular nucleotide concentrations are lower than necessary for replication dynamics (Mathews, 1985; Warner, 1973); 2) in contrast to yeast, overall dNTP pools are almost unchanged after DNA damage in mammals (Hakansson et al., 2006b); 3) nucleotides are highly vulnerable to alterations by ROS (Mathews, 2006) and compartmentalization would provide fresh dNTPs for replication; 4) the RNR was found to travel with the RF in *E.coli* (Sanchez-Romero et al., 2010). The speculations about a role for nuclear RNR were recently fueled by a number of publications reporting nuclear targeting of RNR to sites of DSB (D'Angiolella et al., 2012; Hu et al., 2012; Niida et al., 2010a; Zhang et al., 2009). In contrast to DNA repair, and through several independent approaches such as isolation of proteins on nascent DNA (iPOND), biochemical fractionation or immunofluorescence, we have failed to detect an increase in nuclear RNR in replicating chromatin or even at the RF (data not shown). Those results suggest that the nuclear translocation of the RNR might be specific for DNA break repair and not required during DNA replication or RS. Although it is possible that the DNA damage-induced translocation of the RNR has non-nucleotide reduction related function, this explanation seems unlikely for various reasons. On one hand, the recruitment of the RNR seems to be critical only in the context of low dNTP levels during G0/G1. On the other hand, its reductase activity is essential for efficient DNA repair (Hakansson et al., 2006b; Niida et al., 2010a). Thus, the need for a localized nucleotide production at sites of DNA repair might stem from the low overall dNTP levels in resting cells. The dNTP pool is increased 15-20 times during

S-phase, while DNA damage does not cause major nucleotide pool expansions in mammalian cells (Hakansson et al., 2006b). In this context cells may require a specific mechanism to provide dNTPs for DNA repair while during DNA replication, elevated dNTP levels in the nucleus are sufficient to duplicate the DNA. In support of this hypothesis, RRM2B, the subunit involved in DSB repair, shows a more pronounced nuclear localization compared to RRM2, the subunit providing dNTPs for DNA replication **(Figure 38)**.

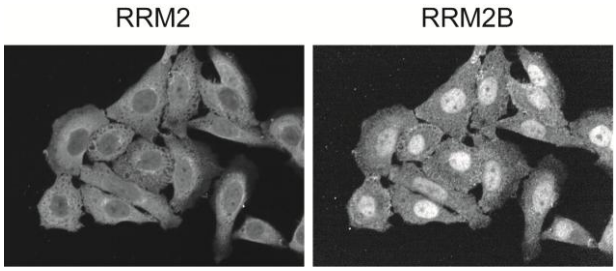


Figure 38: Cellular distribution of RRM2 and RRM2B in U2OS cells.

Besides RRM2B, immunohistochemistry data from *Rrm2*^{Tg} mice show a mainly cytoplasmic distribution of RRM2 in all tissues except for testis, where RRM2 shows a clear nuclear localization **(Figure 39).** Given that the spermatocytes are constitutively exposed to high amounts of DNA breakage (Sasaki and Matsui, 2008), these results suggest that the nuclear localization of RNR might be particularly relevant for cells undergoing active DNA repair.

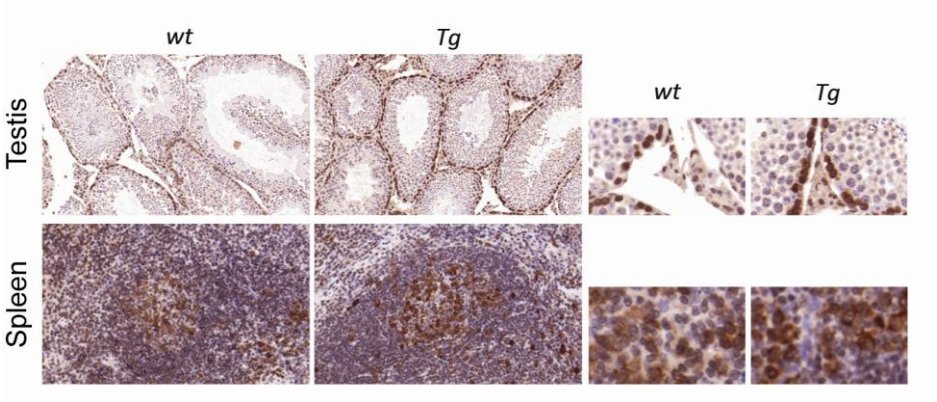


Figure 39: RRM2 levels in tissues of wt and *Rrm2*^{Tg} mice. RRM2 immunohistochemistry of wt and *Rrm2*^{Tg} spleen and testis. Note that whereas RRM2 is cytoplasmic in spleen (and other organs), it is nuclear in testis, in agreement with the increase of nuclear RRM2 levels that has been observed in response to DNA damage (Lopez-Contreras et al., 2015).

Alternatively, the failure to detect the RNR at RFs might be due to technical limitations. In this line, it would be interesting to study whether cells expressing exclusively cytoplasmic RNR subunits in a RNR-deficient background proliferate normally under unchallenged conditions. If so, a role for nuclear RNR during normal replication would be unlikely. In addition, this approach would allow to test whether the potential nuclear localization of the distinct RNR subunits might be independent of the complex and unrelated to dNTP production as previously proposed (Gautam et al., 2003).

Finally and besides the RNR, dNTP synthesis requires multiple enzymes and it is possible that other steps of the dNTP production process act locally at the replisome. Interestingly, the *de novo* thymidylate synthesis pathway forms a multienzyme complex that has been detected at RFs (Hu et al., 2012). Additionally, the nucleotide diphosphate kinase NME1, essential for conversion of dNDPs produced by the RNR into nucleotides, has been found to translocate to the nucleus in response to DNA damage (Kaetzel et al., 2006). Furthermore, NME1 and its homolog NME2 have recently been shown to localize to the RF (Aranda et al., 2014). These results suggest that while the RNR might not be at RFs, the final step of dNTP production in mammals might happen preferentially around sites of DNA replication.

3 Exploring new interactors of the RNR

Besides our interest in ATR biology, we here sought to identify new ways to regulate the mammalian RNR. Through multiple approaches, we provide a comprehensive analysis of proteins potentially binding to the RNR complex. However, none of them could be confirmed as a true interactor, suggesting that the mammalian RNR might not be regulated via other protein inhibitors/activators. Alternatively, ATR may regulate RNR by other means, most prominently through post-translational modifications. In fact, ATM- and ATR-dependent phosphorylations of RRM2B and RRM2 have been implicated in their stabilization in response to DNA damage (Chang et al., 2008; D'Angiolella et al., 2012). However,

whether and how this signaling plays a role during replication and RS remains to be explored. In addition, and although we did not identify any new protein interactors, we cannot exclude that the RNR complex interacts with another protein under certain conditions (see below IRBIT). Taken together, our results indicate that the RNR is a rather stable heterocomplex composed of the RRM1, RRM2 and RRM2B subunits and it does not seem to be regulated by another protein at stoichiometric levels.

4 KPNB1 suppresses RS

A recent paper by Mercedes Dosil reported that the ribosome biogenesis factor Rrp12 is involved in karyopherin (Kap121)-dependent nuclear import of the small RNR subunits Rnr2 and Rnr4 (Dosil, 2011). Defective function of Rrp12/Kap121-dependent nuclear import of Rnr2/Rnr4 led to aberrant dNTP levels, defects in the DNA damage response and S-phase progression. Here, we identify the mammalian karyopherin KPNB1 as a new regulator of the RNR. In agreement with data from yeast, depletion of KPNB1 slows down proliferation and increases RS. In yeast, the defects in karyopherin mutants are rescued by overexpression of Rrp12. In contrast, we find that depletion of human RRP12 does not induce RS and its overexpression does not rescue RS induced by KPNB1-depletion (data not shown). Of note, although we failed to detect any effect of KPNB1 depletion on the distribution of the RNR subunits, we observed an overall reduction of RRM2 and RRM2B levels after downregulation of KPNB1. Whether this is a direct consequence or a secondary effect caused by a perturbation of the cell cycle remains to be explored.

In this work we establish a new function for KPNB1 as a suppressor of RS induced by reduced dNTP levels, opening up the question on how KPNB1 regulates RS levels. We propose two potential mechanisms. First, KPNB1 could directly import RSR proteins into the nucleus, thereby affecting RSR function. Second, KPNB1 could indirectly contribute to the regulation of the dNTP pool through the modulation of the transport of a different substrate. Indeed, KPNB1 can regulate

dNTP levels by mediating the nuclear import of the dNTP nuclease sterile alpha motif (SAM) and HD domain containing 1 (SAMHD1), a step essential for its degradation. Reduced KPNB1 levels lead to a cytosolic accumulation of active SAMHD1 which negatively affects dNTP pool levels (Schaller et al., 2014). Whether this mechanism accounts for the KPNB1-dependent regulation of RS remains to be further investigated **(Figure 40)**.

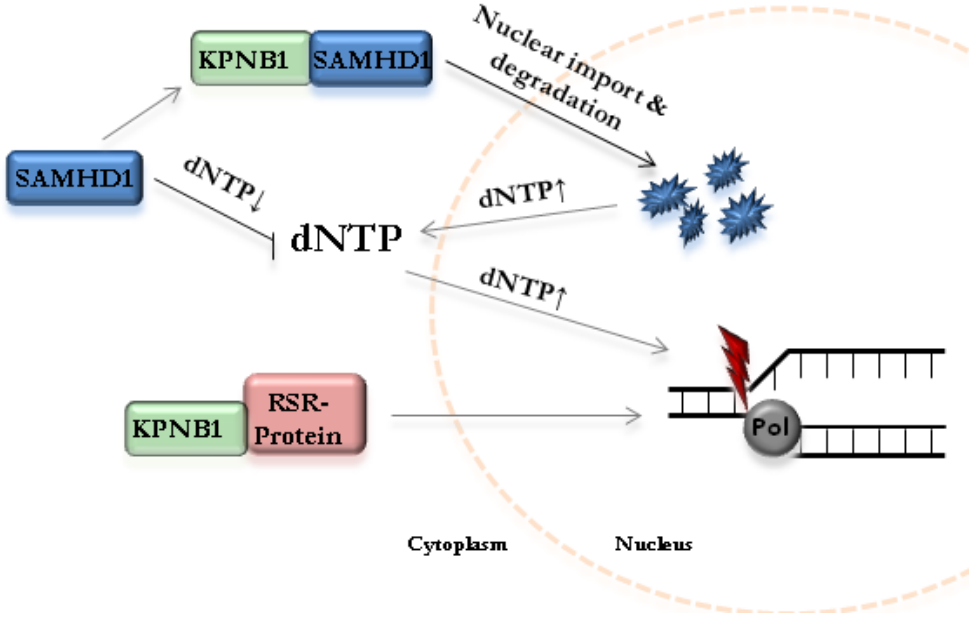


Figure 40: Model for KPNB1-dependent regulation of cellular RS levels. KPNB1 acts on the dNTP pool by mediating nuclear import of SAMHD1 (Schaller et al., 2014). In addition, KPNB1 could regulate RS levels through the nuclear import of an unknown protein of the RSR.

Cancer cells frequently suffer from high levels of oncogene-induced RS caused by forced and untimely hyper-replication. In parallel, RS-promoting oncogenes frequently upregulate proteins of the RSR and dNTP production pathways to cope with these increased levels of RS (Aye et al., 2015; Hoglund et al., 2011; Schulze et al., 2014; Verlinden et al., 2007). This upregulation is often achieved via deregulation of the RB/E2F pathway, a key cell cycle regulator disrupted in almost all human cancers (Reviewed in (Nevins, 2001)). Interestingly, KPNB1 is an E2F target and frequently overexpressed in cancer cells (van der Watt et al., 2011) and tumor cells seem to be particularly dependent on high KPNB1 levels with its

inhibition leading to cell death via apoptosis (van der Watt et al., 2009). Previous studies in our lab revealed that overexpression of the RSR protein and E2F target CHK1 promotes oncogene-induced transformation protecting cancer cells from RS (Lopez-Contreras et al., 2012). Therefore, we propose that KPNB1 overexpression might promote malignant transformation by limiting RS in cancer. In agreement with this hypothesis, KPNB1 was recently identified as a potential anticancer target (van der Watt et al., 2013). Our work suggests that strategies targeting KPNB1 in combination with other RS-inducing drugs such as ATRi might be specifically toxic for tumor cells and could be explored as a therapy for certain types of cancer.

5 An extra supply of dNTPs reduces the pathological consequences of RS

In this work, we demonstrate that addition of nucleosides to cell culture media alleviates RS and growth defects caused by deficient ATR activity. Our results complement a number of recent studies reporting that an exogenous supply of nucleosides reduces the pathological consequences of RS such as growth defects, DNA damage and genomic rearrangements *in vitro*. Bester and colleagues showed in 2011 that the DNA damage caused by oncogene-induced RS could be suppressed by an exogenous supply of nucleosides (Bester et al., 2011). In agreement with this, MEFs transformed by Ras/E1A oncogenes exhibit lower RS and transform more efficiently when nucleosides are added to the medium, suggesting that an increased nucleotide supply helps to promote survival during transformation (our own observations). In addition, reduced RS and increased survival during Ras/E1A-induced transformation was also observed in cells carrying an extra allele of *Chk1* (Lopez-Contreras et al., 2012), supporting the idea that ATR/CHK1 signaling and nucleoside supplementation have similar consequences. Analogous to oncogene-induced RS, we recently described reprogramming-induced RS as a source for genomic instability in induced pluripotent stem (iPS) cells (Ruiz, 2015). Addition of nucleosides during somatic cell reprogramming alleviates RS, as

observed after oncogene-induced transformation, which leads to reduced genomic rearrangements on the resultant iPS. Together, those results show that stimulation of dNTP production reduces the pathological consequences of RS *in vitro* and establish dNTPs scarcity as a key source for RS.

The DNA damage and genomic instability derived from RS are known causes for cancer and aging in humans (Lecona and Fernandez-Capetillo, 2014). This opens up the question as to whether a stimulation of dNTP production can also lower the pathological consequences of RS *in vivo*. Regarding the role of increased dNTP supply during cancer development, the data is controversial and points towards a dose and stage specific effect. A previous report showed that widespread overexpression of the small RNR subunits potently and selectively induces Non small cell lung cancer (NSCLC) in transgenic mice and is mutagenic in cultured cells (Xu et al., 2008). In contrast, we did not observe enhanced tumor incidence in *Rrm2*^{Tg} mice. One possible explanation for this discrepancy is that we expressed RRM2 under its endogenous promoter, avoiding ectopic overexpression in places where RRM2 is usually not expressed, such as the lung. To further clarify this issue and given the fact that RNR-induced lung neoplasms were associated with K-ras proto-oncogene mutations, we crossed *Rrm2*^{Tg} mice with a genetic model of lung-carcinogenesis induced by the K-Ras oncogene (Guerra et al., 2003). Again, we failed to detect a higher incidence of tumors in this model. We conclude that increased RNR activity in *Rrm2*^{Tg} mice does not promote tumor formation. In contrast, increased RNR activity reduces RS and diminishes the severity of pathologies associated with ATR-Seckel, namely premature aging and death. Hence, reduced dNTP levels are a main cause for ATR deficient phenotypes in mammals and an increased supply of dNTPs can indeed reduce RS-derived pathologies in mice. Interestingly, folates, essential for *de novo* synthesis of thymidine and purines, are routinely used in the clinic for the prevention or treatment of a wide range of age-associated diseases, including cancer. Low folate levels seem to play an important role in ageing brain processes like Alzheimer's disease and vascular dementia (Reynolds, 2002) as well as during tumor

development (Kim, 1999), and higher intake of folates in the diet correlates with reduced risk for those diseases (Ericson et al., 2007; Mason, 2011). However, it remains unclear whether folate supplementation is only beneficial in the context of folate deficiency, which is particularly common among elderly people, and some concerns have been raised regarding an excess intake of folates (Kim, 2004). Nevertheless, the data from mice and human suggest that an enhancement of dNTP metabolism has beneficial effects over cancer development and other age-related diseases in mammals, as long as it stays below a certain threshold **(Figure 41).** In this context, it would be interesting to explore whether increased dNTP levels could also rescue other types of genomic instability diseases as has recently been described for yeast (Poli et al., 2012). The *Rrm2*^{Tg} mouse provides a valuable model of enhanced RNR activity and could be used to further study the impact of increased RNR activity on other genomic instability driven diseases. In any case, the work presented here supports that evaluating whether and which types of medical conditions could benefit from increased dNTP levels demands further investigation.

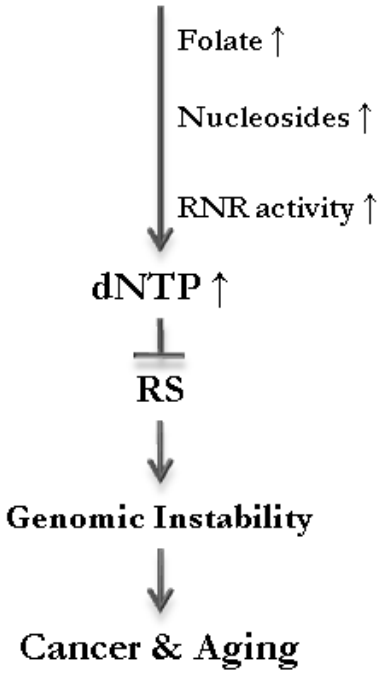


Figure 41: Increased levels of dNTPs help to reduce cancer and aging in mammals. Elevated dNTP levels due to a stimulation of dNTP synthesis lead to reduced RS, which results in reduced genomic instability affecting cancer and aging *in vivo*.

5.1 How does limited ATR activity relate to dNTP pools?

An obvious remaining question of our work is, why are the dNTP pools decreased in ATR-Seckel mice? Is this directly caused by reduced RNR activity like in yeast *mec1Δ* mutants? What are the essential mechanisms linking ATR and RNR? On one hand, ATR can regulate the transcription of RRM2. Upon DNA damage, ATR has also been shown to limit the degradation of RRM2 (D'Angiolella et al., 2012). However, this mechanism has only been linked to DNA damage and limited to G2 and cannot explain how ATR regulates RNR during replication and RS. Further, ATR deficiency leads to increased origin firing (Paulsen and Cimprich, 2007) and causes an exhaustion of the dNTP pool. Therefore, increased RNR activity might provide sufficient dNTPs under conditions of increased demand, such as when multiple origins are fired. Based on the homology to yeast, we think that yet another regulatory mechanism might exist, namely, the regulation of RNR activity by ATR. In this context, it would be highly valuable to develop a new method to directly measure local RNR activity. So far, RNR activity is evaluated by measuring dNTP levels, a technically complicated and rather imprecise method detecting whole cell nucleotide levels. The development of an alternative activity assay would allow to study the connection between ATR signaling and RNR activity and could further help in the search for factors that induce RNR activity. Then, strategies to increase the activity of RNR could be further explored for a potential therapeutic use in certain types of human diseases (see above).

6 Limiting RNR function in vivo: the search for a mammalian Sml1

We here show that the RRM1W684G point mutation compromises RRM1 function in mice, yet, the mechanism differs from that reported in yeast. In mice, the RRM1-W684G mutation prevents the RRM1-RRM2(B) interaction and leads to early embryonic lethality. Interestingly, recombinant RRM1-WG interacts normally with RRM2(B) *in vitro*, which raises the question of why the RNR complex does not form *in vivo* with RRM1-WG. One option is that post-translational modifications that

occur *in vivo* are necessary to prevent the interaction. Alternatively, RRM1-WG could bind another protein *in vivo* blocking RRM1 binding to RRM2(B). In accordance with this hypothesis, studies with recombinant mammalian RNR suggest that binding of yeast Sml1 to RRM1 interferes with binding to RRM2 (Chabes et al., 1999). Unfortunately, we have been unable to find a factor with increased affinity for RRM1-WG in mammalian cells. A third explanation for the loss of the RRM1-WG-RRM2(B) interaction relies on the suggested function of the sequence surrounding the point mutation. In yeast, this region mediates the interaction with the neighbouring Rnr1-CTD, an essential step required for complex regeneration (Zhang et al., 2007). Sml1 binds to this region and outcompetes Rnr1-CTD, preventing the regeneration of active RNR. Because the RRM1 sequence is highly conserved, it is also possible that the point mutation induces constitutive binding of the neighbouring Rnr1-CTD, impeding reactivation of the RNR and interfering with the binding to RRM2(B) **(Figure 40).** Regardless of the mechanism, the fact that the mutant protein fails to participate in RNR complexes explains the early embryonic lethality as well as the absence of toxic effects of RRM1-WG even when overexpressed. In conclusion, our study shows that the conserved RRM1-WG residue is essential for the formation of a functional RNR complex in mammals and that this step is essential for mammalian cellular viability.

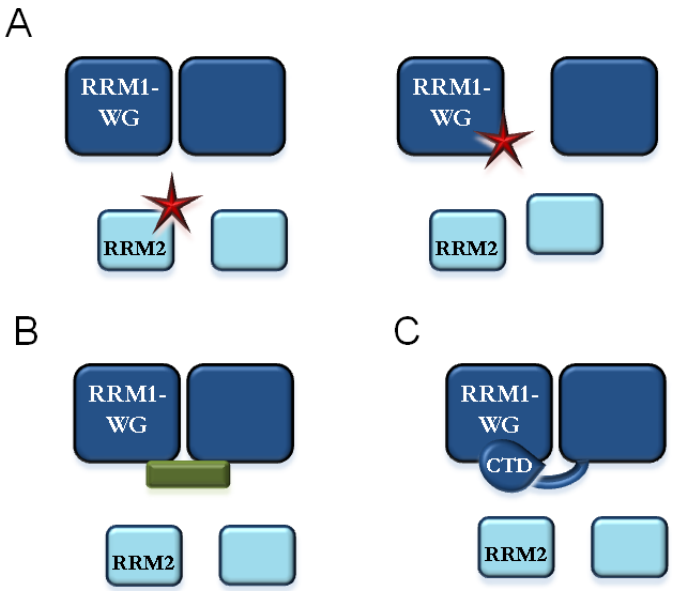


Figure 40: Potential mechanisms interfering with RRM1-WG binding to RRM2(b) *in vivo*. (A) The formation of the RNR heterocomplex or RRM1 homodimer might be compromised by protein modifications (red star) e.g. on RRM2(b) or RRM1. (B) Alternatively, binding of an unknown protein to RRM1-WG might interfere with complex formation (green rectangle). (C) Irreversible interaction of RRM1-CTD to the neighbouring RRM1-WG could impede binding of the small subunits.

In what regards to the identification of a mammalian Sml1, proteomic analyses failed to detect any protein that binds more avidly to RRM1 carrying the W684G mutation than to wt RRM1. A recent study proposed IRBIT as a mammalian ortholog for Sml1 that interacts with the RRM1-RRM2(B) complex (Arnaoutov and Dasso, 2014). In contrast, we did not detect any interaction between IRBIT and RRM1 or RRM1-WG, what might be explained by the fact that the IRBIT-RNR interaction seems to be limited to mitosis. To further clarify whether IRBIT might act as a mammalian ortholog of Sml1, we are currently crossing IRBIT knock out mice with ATR-Seckel mice. If IRBIT were to act homologous to Sml1, this cross should rescue the pathologies associated with ATR-deficiency, just as *mec1Δ* can be rescued by depleting Sml1 (Zhao et al., 1998). In addition, we are now conducting a genome wide screening for genes that, when knocked out, confer resistance to ATR inhibition. We hope that these experiments might shed further light on the essential functions of ATR in mammals.

6.1 Protein levels of RRM1 are not limiting in mammals

In humans, the *Rrm1* gene lies at 11p15.5, a tumor suppressor region (Pitterle et al., 1999) and RRM1 expression levels have been correlated with DNA repair capacity in response to certain chemotherapeutic drugs (Aye et al., 2015). Accordingly, the levels of RRM1 have been proposed to predict sensitivity to chemotherapy in pancreatic and non-small cell lung cancers, although conflicting data exist (Besse et al., 2013). Here we show that having one functional RRM1 is sufficient for an efficient RS response, normal mouse development and organ function, which argues against the view that RRM1 levels would be reliable predictors of response to treatment. So far, we failed to detect an increased tumor incidence in mice with reduced wt RRM1 levels further arguing against a major role for RRM1 heterozygosity in tumor suppression. Together, those results suggest that RRM1 exists in large excess in mammalian cells, and that RNR function mainly depends on the availability of RRM2. As a consequence, a reduction of RRM1 levels to as much as 50% would have negligible effects on RNR function. This finding has important implications for the clinic as it casts doubt on the utility of RRM1 levels as marker for resistance to treatment. In contrast, our data suggest that any changes in RRM1 levels, to be considered meaningful as guidance for treatment, should reduce RRM1 expression below 50%.

In conclusion, we present the characterization of the first loss-of-function mouse model of the RNR, reveal a single amino acid change that is essential for life in mammals and show that RRM1 levels are not limiting for RNR function, which would mostly depend on the presence of RRM2.

CONCLUSIONS

ATR and the RNR

1. ATR activity stimulates the expression of RRM1 and RRM2.
2. Nucleoside supplementation reduces the RS and growth deficiencies of ATR-Seckel cells.
3. Increased RRM2 levels reduce the severity of the symptoms found on ATR-Seckel mice and significantly extend their lifespan.

New Interactors of the RNR

4. KPNB1 (importin subunit beta-1), is identified as a putative new regulator of the mammalian RNR.
5. KPNB1 overexpression reduces, and depletion augments, RS in human cells.
6. Proteomic analyses on purified RNR complexes failed to identify any factor that interacts with the mammalian RNR at a ratio similar to RRM1, RRM2 or RRM2B.

A new mouse model of RRM1

7. A single aminoacid change in RRM1 is incompatible with life in mice.
8. *Rrm1*^{WG} heterozygosity does not affect the function of the RNR nor has a detectable impact in mice.
9. Proteomic analyses failed to identify any factor that binds more avidly to RRM1-W684G than to wild type RRM1.
10. Whereas recombinant RRM1-W684G interacts with RRM2 *in vitro*, it fails to do so *in vivo*, providing an explanation for the essential nature of the mutation.

11. The absence of phenotypes on *Rrm1*^{WG} heterozygous mice indicates that RRM1 levels are largely in excess in mammalian cells.

CONCLUSIONES

ATR y la RNR

1. La actividad de ATR induce la expresión de RRM1 y RRM2.
2. El suplemento con nucleósidos reduce el estrés replicativo y los defectos en el crecimiento de las células ATR-Seckel.
3. Niveles elevados de RRM2 disminuyen la gravedad de los síntomas que se manifiestan en los ratones ATR-Seckel e incrementan significativamente su esperanza de vida.

Nuevos interactores de la RNR

4. La KPNB1 (subunidad beta-1 de la importina) es identificada como un presunto nuevo regulador de la RNR en mamíferos.
5. La sobreexpresión de KPNB1 reduce, y su eliminación aumenta, el estrés replicativo en células humanas.
6. El análisis proteómico de complejos purificados de RNR no logró identificar ningún factor que interaccione con la RNR de mamíferos en proporciones similares a las que presentan RRM1, RRM2 o RRM2B.

Un nuevo modelo murino de RRM1

7. El cambio de un solo aminoácido en RRM1 es incompatible con la vida en ratones.
8. *Rrm1*^{WG} en heterocigosis no afecta a la función de RNR ni tiene ningún impacto detectable en ratones.
9. El análisis proteómico no permitió identificar ningún factor que se una con mayor afinidad a RRM1-W684G que a la forma silvestre de RRM1.

10. Mientras que la proteína recombinante RRM1-W684G interacciona con RRM2 *in vitro*, no es capaz de hacerlo *in vivo*, proporcionando así una explicación a la naturaleza esencial de la mutación.
11. La ausencia de fenotipos en ratones heterocigotos para *Rrm1*^{WG} indica que RRM1 se encuentra ampliamente en exceso en las células de mamíferos.

BIBLIOGRAPHY

Ahmad, M.F., and Dealwis, C.G. (2013). The structural basis for the allosteric regulation of ribonucleotide reductase. Prog Mol Biol Transl Sci *117*, 389-410.

Aird, K.M., and Zhang, R. (2015). Nucleotide metabolism, oncogene-induced senescence and cancer. Cancer letters *356*, 204-210.

Akerblom, L., Ehrenberg, A., Graslund, A., Lankinen, H., Reichard, P., and Thelander, L. (1981). Overproduction of the free radical of ribonucleotide reductase in hydroxyurea-resistant mouse fibroblast 3T6 cells. Proceedings of the National Academy of Sciences of the United States of America *78*, 2159-2163.

Alvarez-Salas, L.M. (2008). Nucleic acids as therapeutic agents. Current topics in medicinal chemistry *8*, 1379-1404.

Alvino, G.M., Collingwood, D., Murphy, J.M., Delrow, J., Brewer, B.J., and Raghuraman, M.K. (2007). Replication in hydroxyurea: it's a matter of time. Molecular and cellular biology *27*, 6396-6406.

Andreson, B.L., Gupta, A., Georgieva, B.P., and Rothstein, R. (2010). The ribonucleotide reductase inhibitor, Sml1, is sequentially phosphorylated, ubiquitylated and degraded in response to DNA damage. Nucleic acids research *38*, 6490-6501.

Anglana, M., Apiou, F., Bensimon, A., and Debatisse, M. (2003). Dynamics of DNA replication in mammalian somatic cells: nucleotide pool modulates origin choice and interorigin spacing. Cell *114*, 385-394.

Angus, S.P., Wheeler, L.J., Ranmal, S.A., Zhang, X., Markey, M.P., Mathews, C.K., and Knudsen, E.S. (2002). Retinoblastoma tumor suppressor targets dNTP metabolism to regulate DNA replication. The Journal of biological chemistry *277*, 44376-44384.

Aranda, S., Rutishauser, D., and Ernfors, P. Identification of a large protein network involved in epigenetic transmission in replicating DNA of embryonic stem cells. Nucleic Acids Res *42*, 6972-6986.

Aranda, S., Rutishauser, D., and Ernfors, P. (2014). Identification of a large protein network involved in epigenetic transmission in replicating DNA of embryonic stem cells. Nucleic acids research *42*, 6972-6986.

Aravind, L., Wolf, Y.I., and Koonin, E.V. (2000). The ATP-cone: an evolutionarily mobile, ATP-binding regulatory domain. J Mol Microbiol Biotechnol *2*, 191-194.

Arlt, M.F., Ozdemir, A.C., Birkeland, S.R., Wilson, T.E., and Glover, T.W. Hydroxyurea induces de novo copy number variants in human cells. Proceedings of the National Academy of Sciences of the United States of America *108*, 17360-17365.

Arlt, M.F., Ozdemir, A.C., Birkeland, S.R., Wilson, T.E., and Glover, T.W. (2011). Hydroxyurea induces de novo copy number variants in human cells. Proceedings of the National Academy of Sciences of the United States of America *108*, 17360-17365.

Arnaoutov, A., and Dasso, M. (2014). Enzyme regulation. IRBIT is a novel regulator of ribonucleotide reductase in higher eukaryotes. Science *345*, 1512-1515.

Arner, E.S., and Eriksson, S. (1995). Mammalian deoxyribonucleoside kinases. Pharmacol Ther *67*, 155-186.

Artin, E., Wang, J., Lohman, G.J., Yokoyama, K., Yu, G., Griffin, R.G., Bar, G., and Stubbe, J. (2009). Insight into the mechanism of inactivation of ribonucleotide reductase by gemcitabine 5'-diphosphate in the presence or absence of reductant. Biochemistry *48*, 11622-11629.

Austin, W.R., Armijo, A.L., Campbell, D.O., Singh, A.S., Hsieh, T., Nathanson, D., Herschman, H.R., Phelps, M.E., Witte, O.N., Czernin, J.*, et al.* (2012). Nucleoside salvage pathway kinases regulate hematopoiesis by linking nucleotide metabolism with replication stress. The Journal of experimental medicine *209*, 2215-2228.

Aye, Y., Li, M., Long, M.J.C., and Weiss, R.S. (2015). Ribonucleotide reductase and cancer: biological mechanisms and targeted therapies. Oncogene *34*, 2011-2021.

Besse, B., Olaussen, K.A., and Soria, J.C. ERCC1 and RRM1: ready for prime time? J Clin Oncol *31*, 1050-1060.

Besse, B., Olaussen, K.A., and Soria, J.C. (2013). ERCC1 and RRM1: ready for prime time? Journal of clinical oncology : official journal of the American Society of Clinical Oncology *31*, 1050-1060.

Bester, A.C., Roniger, M., Oren, Y.S., Im, M.M., Sarni, D., Chaoat, M., Bensimon, A., Zamir, G., Shewach, D.S., and Kerem, B. Nucleotide deficiency promotes genomic instability in early stages of cancer development. Cell *145*, 435-446.

Bester, A.C., Roniger, M., Oren, Y.S., Im, M.M., Sarni, D., Chaoat, M., Bensimon, A., Zamir, G., Shewach, D.S., and Kerem, B. (2011). Nucleotide deficiency promotes genomic instability in early stages of cancer development. Cell *145*, 435-446.

Bianchi, V., Pontis, E., and Reichard, P. (1986). Changes of deoxyribonucleoside triphosphate pools induced by hydroxyurea and their relation to DNA synthesis. The Journal of biological chemistry *261*, 16037-16042.

Bjorklund, S., Skog, S., Tribukait, B., and Thelander, L. (1990). S-phase-specific expression of mammalian ribonucleotide reductase R1 and R2 subunit mRNAs. Biochemistry *29*, 5452-5458.

Bonate, P.L., Arthaud, L., Cantrell, W.R., Jr., Stephenson, K., Secrist, J.A., 3rd, and Weitman, S. (2006). Discovery and development of clofarabine: a nucleoside analogue for treating cancer. Nat Rev Drug Discov *5*, 855-863.

Brown, E.J., and Baltimore, D. (2000). ATR disruption leads to chromosomal fragmentation and early embryonic lethality. Genes & development *14*, 397-402.

Brown, E.J., and Baltimore, D. (2003). Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. Genes & development *17*, 615-628.

Brown, N.C., and Reichard, P. (1969). Ribonucleoside diphosphate reductase. Formation of active and inactive complexes of proteins B1 and B2. J Mol Biol *46*, 25-38.

Byun, T.S., Pacek, M., Yee, M.C., Walter, J.C., and Cimprich, K.A. (2005). Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. Genes & development *19*, 1040-1052.

Cao, M.Y., Lee, Y., Feng, N.P., Xiong, K., Jin, H., Wang, M., Vassilakos, A., Viau, S., Wright, J.A., and Young, A.H. (2003). Adenovirus-mediated ribonucleotide reductase R1 gene therapy of human colon adenocarcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research *9*, 4553-4561.

Cimprich, K.A., and Cortez, D. (2008). ATR: an essential regulator of genome integrity. Nat Rev Mol Cell Biol *9*, 616-627.

Cortez, D., Guntuku, S., Qin, J., and Elledge, S.J. (2001). ATR and ATRIP: partners in checkpoint signaling. Science *294*, 1713-1716.

Cotruvo, J.A., and Stubbe, J. (2011). Class I ribonucleotide reductases: metallocofactor assembly and repair in vitro and in vivo. Annu Rev Biochem *80*, 733-767.

Courbet, S., Gay, S., Arnoult, N., Wronka, G., Anglana, M., Brison, O., and Debatisse, M. (2008). Replication fork movement sets chromatin loop size and origin choice in mammalian cells. Nature *455*, 557-560.

Chabes, A., Domkin, V., Larsson, G., Liu, A., Graslund, A., Wijmenga, S., and Thelander, L. (2000). Yeast ribonucleotide reductase has a heterodimeric iron-radical-containing subunit. Proceedings of the National Academy of Sciences of the United States of America *97*, 2474-2479.

Chabes, A., Domkin, V., and Thelander, L. (1999). Yeast Sml1, a protein inhibitor of ribonucleotide reductase. The Journal of biological chemistry *274*, 36679-36683.

Chabes, A., Georgieva, B., Domkin, V., Zhao, X., Rothstein, R., and Thelander, L. (2003a). Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase. Cell *112*, 391-401.

Chabes, A., and Thelander, L. (2000). Controlled protein degradation regulates ribonucleotide reductase activity in proliferating mammalian cells during the normal cell cycle and in response to DNA damage and replication blocks. The Journal of biological chemistry *275*, 17747-17753.

Chabes, A., and Thelander, L. (2003). DNA building blocks at the foundation of better survival. Cell cycle *2*, 171-173.

Chabes, A.L., Bjorklund, S., and Thelander, L. (2004). S Phase-specific transcription of the mouse ribonucleotide reductase R2 gene requires both a proximal repressive E2F-binding site and an upstream promoter activating region. The Journal of biological chemistry *279*, 10796-10807.

Chabes, A.L., Pfleger, C.M., Kirschner, M.W., and Thelander, L. (2003b). Mouse ribonucleotide reductase R2 protein: a new target for anaphase-promoting complex-Cdh1-mediated proteolysis. Proceedings of the National Academy of Sciences of the United States of America *100*, 3925-3929.

Chang, L., Zhou, B., Hu, S., Guo, R., Liu, X., Jones, S.N., and Yen, Y. (2008). ATM-mediated serine 72 phosphorylation stabilizes ribonucleotide reductase small subunit p53R2 protein against MDM2 to DNA damage. Proceedings of the National Academy of Sciences of the United States of America *105*, 18519-18524.

D'Angiolella, V., Donato, V., Forrester, F.M., Jeong, Y.T., Pellacani, C., Kudo, Y., Saraf, A., Florens, L., Washburn, M.P., and Pagano, M. (2012). Cyclin F-mediated degradation of ribonucleotide reductase M2 controls genome integrity and DNA repair. Cell *149*, 1023-1034.

Danilova, N., Bibikova, E., Covey, T.M., Nathanson, D., Dimitrova, E., Konto, Y., Lindgren, A., Glader, B., Radu, C.G., Sakamoto, K.M., *et al.* (2014). The role of the DNA damage response in zebrafish and cellular models of Diamond Blackfan anemia. Dis Model Mech *7*, 895-905.

Davidson, M.B., Katou, Y., Keszthelyi, A., Sing, T.L., Xia, T., Ou, J., Vaisica, J.A., Thevakumaran, N., Marjavaara, L., Myers, C.L., *et al.* (2012). Endogenous DNA replication stress results in expansion of dNTP pools and a mutator phenotype. The EMBO journal *31*, 895-907.

De Bont, R., and van Larebeke, N. (2004). Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis *19*, 169-185.

de Bruin, R.A., and Wittenberg, C. (2009). All eukaryotes: before turning off G1-S transcription, please check your DNA. Cell cycle *8*, 214-217.

DeGregori, J., Kowalik, T., and Nevins, J.R. (1995). Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. Molecular and cellular biology *15*, 4215-4224.

Dehe, P.M., Coulon, S., Scaglione, S., Shanahan, P., Takedachi, A., Wohlschlegel, J.A., Yates, J.R., 3rd, Llorente, B., Russell, P., and Gaillard, P.H. Regulation of Mus81-Eme1 Holliday junction resolvase in response to DNA damage. Nature structural & molecular biology *20*, 598-603.

Dehe, P.M., Coulon, S., Scaglione, S., Shanahan, P., Takedachi, A., Wohlschlegel, J.A., Yates, J.R., 3rd, Llorente, B., Russell, P., and Gaillard, P.H. (2013). Regulation of Mus81-Eme1 Holliday junction resolvase in response to DNA damage. Nature structural & molecular biology *20*, 598-603.

Desler, C., Munch-Petersen, B., Stevnsner, T., Matsui, S., Kulawiec, M., Singh, K.K., and Rasmussen, L.J. (2007). Mitochondria as determinant of nucleotide pools and chromosomal stability. Mutat Res *625*, 112-124.

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre, M., Nuciforo, P.G., Bensimon, A.*, et al.* (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature *444*, 638-642.

Dominguez-Sola, D., Ying, C.Y., Grandori, C., Ruggiero, L., Chen, B., Li, M., Galloway, D.A., Gu, W., Gautier, J., and Dalla-Favera, R. (2007). Non-transcriptional control of DNA replication by c-Myc. Nature *448*, 445-451.

Dosil, M. (2011). Ribosome synthesis-unrelated functions of the preribosomal factor Rrp12 in cell cycle progression and the DNA damage response. Molecular and cellular biology *31*, 2422-2438.

Duxbury, M.S., Ito, H., Benoit, E., Zinner, M.J., Ashley, S.W., and Whang, E.E. (2004a). Retrovirally mediated RNA interference targeting the M2 subunit of ribonucleotide reductase: A novel therapeutic strategy in pancreatic cancer. Surgery *136*, 261-269.

Duxbury, M.S., Ito, H., Zinner, M.J., Ashley, S.W., and Whang, E.E. (2004b). RNA interference targeting the M2 subunit of ribonucleotide reductase enhances pancreatic adenocarcinoma chemosensitivity to gemcitabine. Oncogene *23*, 1539-1548.

Eaton, J.S., Lin, Z.P., Sartorelli, A.C., Bonawitz, N.D., and Shadel, G.S. (2007). Ataxia-telangiectasia mutated kinase regulates ribonucleotide reductase and mitochondrial homeostasis. J Clin Invest *117*, 2723-2734.

Elford, H.L., Freese, M., Passamani, E., and Morris, H.P. (1970). Ribonucleotide reductase and cell proliferation. I. Variations of ribonucleotide reductase activity with tumor growth rate in a series of rat hepatomas. The Journal of biological chemistry *245*, 5228-5233.

Elledge, S.J. (1996). Cell cycle checkpoints: preventing an identity crisis. Science *274*, 1664-1672.

Engstrom, Y., Eriksson, S., Jildevik, I., Skog, S., Thelander, L., and Tribukait, B. (1985). Cell cycle-dependent expression of mammalian ribonucleotide reductase. Differential regulation of the two subunits. The Journal of biological chemistry *260*, 9114-9116.

Engstrom, Y., and Rozell, B. (1988). Immunocytochemical evidence for the cytoplasmic localization and differential expression during the cell cycle of the M1 and M2 subunits of mammalian ribonucleotide reductase. The EMBO journal *7*, 1615-1620.

Ericson, U., Sonestedt, E., Gullberg, B., Olsson, H., and Wirfalt, E. (2007). High folate intake is associated with lower breast cancer incidence in postmenopausal women in the Malmo Diet and Cancer cohort. The American journal of clinical nutrition *86*, 434-443.

Fan, H., Huang, A., Villegas, C., and Wright, J.A. (1997). The R1 component of mammalian ribonucleotide reductase has malignancy-suppressing activity as demonstrated by gene

transfer experiments. Proceedings of the National Academy of Sciences of the United States of America *94*, 13181-13186.

Fan, H., Villegas, C., Huang, A., and Wright, J.A. (1998). The mammalian ribonucleotide reductase R2 component cooperates with a variety of oncogenes in mechanisms of cellular transformation. Cancer research *58*, 1650-1653.

Fan, H., Villegas, C., and Wright, J.A. (1996). Ribonucleotide reductase R2 component is a novel malignancy determinant that cooperates with activated oncogenes to determine transformation and malignant potential. Proceedings of the National Academy of Sciences of the United States of America *93*, 14036-14040.

Farber, S., and Diamond, L.K. (1948). Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4-aminopteroyl-glutamic acid. The New England journal of medicine *238*, 787-793.

Filatov, D., Bjorklund, S., Johansson, E., and Thelander, L. (1996). Induction of the mouse ribonucleotide reductase R1 and R2 genes in response to DNA damage by UV light. The Journal of biological chemistry *271*, 23698-23704.

Filatov, D., and Thelander, L. (1995). Role of a proximal NF-Y binding promoter element in S phase-specific expression of mouse ribonucleotide reductase R2 gene. The Journal of biological chemistry *270*, 25239-25243.

Finch, R.A., Liu, M., Grill, S.P., Rose, W.C., Loomis, R., Vasquez, K.M., Cheng, Y., and Sartorelli, A.C. (2000). Triapine (3-aminopyridine-2-carboxaldehyde- thiosemicarbazone): A potent inhibitor of ribonucleotide reductase activity with broad spectrum antitumor activity. Biochemical pharmacology *59*, 983-991.

Flach, J., Bakker, S.T., Mohrin, M., Conroy, P.C., Pietras, E.M., Reynaud, D., Alvarez, S., Diolaiti, M.E., Ugarte, F., Forsberg, E.C., *et al.* Replication stress is a potent driver of functional decline in ageing haematopoietic stem cells. Nature *512*, 198-202.

Flach, J., Bakker, S.T., Mohrin, M., Conroy, P.C., Pietras, E.M., Reynaud, D., Alvarez, S., Diolaiti, M.E., Ugarte, F., Forsberg, E.C., *et al.* (2014). Replication stress is a potent driver of functional decline in ageing haematopoietic stem cells. Nature *512*, 198-202.

Friedel, A.M., Pike, B.L., and Gasser, S.M. (2009). ATR/Mec1: coordinating fork stability and repair. Curr Opin Cell Biol *21*, 237-244.

Fu, Y., Long, M.J., Rigney, M., Parvez, S., Blessing, W.A., and Aye, Y. (2013). Uncoupling of allosteric and oligomeric regulation in a functional hybrid enzyme constructed from Escherichia coli and human ribonucleotide reductase. Biochemistry *52*, 7050-7059.

Gagou, M.E., Zuazua-Villar, P., and Meuth, M. Enhanced H2AX phosphorylation, DNA replication fork arrest, and cell death in the absence of Chk1. Molecular biology of the cell *21*, 739-752.

Gagou, M.E., Zuazua-Villar, P., and Meuth, M. (2010). Enhanced H2AX phosphorylation, DNA replication fork arrest, and cell death in the absence of Chk1. Molecular biology of the cell *21*, 739-752.

Gautam, A., and Bepler, G. (2006). Suppression of lung tumor formation by the regulatory subunit of ribonucleotide reductase. Cancer research *66*, 6497-6502.

Gautam, A., Li, Z.R., and Bepler, G. (2003). RRM1-induced metastasis suppression through PTEN-regulated pathways. Oncogene *22*, 2135-2142.

Ge, X.Q., Jackson, D.A., and Blow, J.J. (2007). Dormant origins licensed by excess Mcm2-7 are required for human cells to survive replicative stress. Genes & development *21*, 3331-3341.

Georgieva, B., Zhao, X., and Rothstein, R. (2000). Damage response and dNTP regulation: the interaction between ribonucleotide reductase and its inhibitor, Sml1. Cold Spring Harb Symp Quant Biol *65*, 343-346.

Goan, Y.G., Zhou, B., Hu, E., Mi, S., and Yen, Y. (1999). Overexpression of ribonucleotide reductase as a mechanism of resistance to 2,2-difluorodeoxycytidine in the human KB cancer cell line. Cancer research *59*, 4204-4207.

Gonzalez, H., Leblond, V., Azar, N., Sutton, L., Gabarre, J., Binet, J.L., Vernant, J.P., and Dighiero, G. (1998). Severe autoimmune hemolytic anemia in eight patients treated with fludarabine. Hematology and cell therapy *40*, 113-118.

Guerra, C., Mijimolle, N., Dhawahir, A., Dubus, P., Barradas, M., Serrano, M., Campuzano, V., and Barbacid, M. (2003). Tumor induction by an endogenous K-ras oncogene is highly dependent on cellular context. Cancer Cell *4*, 111-120.

Hakansson, P., Dahl, L., Chilkova, O., Domkin, V., and Thelander, L. (2006a). The Schizosaccharomyces pombe replication inhibitor Spd1 regulates ribonucleotide reductase activity and dNTPs by binding to the large Cdc22 subunit. The Journal of biological chemistry *281*, 1778-1783.

Hakansson, P., Hofer, A., and Thelander, L. (2006b). Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. The Journal of biological chemistry *281*, 7834-7841.

Harrison, J.C., and Haber, J.E. (2006). Surviving the breakup: the DNA damage checkpoint. Annu Rev Genet *40*, 209-235.

Hehlmann, R., Grimwade, D., Simonsson, B., Apperley, J., Baccarani, M., Barbui, T., Barosi, G., Bassan, R., Bene, M.C., Berger, U.*, et al.* (2011). The European LeukemiaNet: achievements and perspectives. Haematologica *96*, 156-162.

Hofer, A., Crona, M., Logan, D.T., and Sjoberg, B.M. (2012). DNA building blocks: keeping control of manufacture. Crit Rev Biochem Mol Biol *47*, 50-63.

Hoglund, A., Nilsson, L.M., Muralidharan, S.V., Hasvold, L.A., Merta, P., Rudelius, M., Nikolova, V., Keller, U., and Nilsson, J.A. (2011). Therapeutic implications for the induced

levels of Chk1 in Myc-expressing cancer cells. Clinical cancer research : an official journal of the American Association for Cancer Research *17*, 7067-7079.

Hu, C.M., Yeh, M.T., Tsao, N., Chen, C.W., Gao, Q.Z., Chang, C.Y., Lee, M.H., Fang, J.M., Sheu, S.Y., Lin, C.J., *et al.* (2012). Tumor cells require thymidylate kinase to prevent dUTP incorporation during DNA repair. Cancer Cell *22*, 36-50.

Huang, M., and Elledge, S.J. (1997). Identification of RNR4, encoding a second essential small subunit of ribonucleotide reductase in Saccharomyces cerevisiae. Molecular and cellular biology *17*, 6105-6113.

Huang, M., Zhou, Z., and Elledge, S.J. (1998). The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. Cell *94*, 595-605.

Johansson, E., Hjortsberg, K., and Thelander, L. (1998). Two YY-1-binding proximal elements regulate the promoter strength of the TATA-less mouse ribonucleotide reductase R1 gene. The Journal of biological chemistry *273*, 29816-29821.

Jones, R.M., Mortusewicz, O., Afzal, I., Lorvellec, M., Garcia, P., Helleday, T., and Petermann, E. (2013). Increased replication initiation and conflicts with transcription underlie Cyclin E-induced replication stress. Oncogene *32*, 3744-3753.

Julias, J.G., and Pathak, V.K. (1998). Deoxyribonucleoside triphosphate pool imbalances in vivo are associated with an increased retroviral mutation rate. J Virol *72*, 7941-7949.

Kaetzel, D.M., Zhang, Q., Yang, M., McCorkle, J.R., Ma, D., and Craven, R.J. (2006). Potential roles of 3'-5' exonuclease activity of NM23-H1 in DNA repair and malignant progression. J Bioenerg Biomembr *38*, 163-167.

Kim, Y.I. (1999). Folate and carcinogenesis: evidence, mechanisms, and implications. The Journal of nutritional biochemistry *10*, 66-88.

Kim, Y.I. (2004). Folate, colorectal carcinogenesis, and DNA methylation: lessons from animal studies. Environmental and molecular mutagenesis *44*, 10-25.

King, A.E., Ackley, M.A., Cass, C.E., Young, J.D., and Baldwin, S.A. (2006). Nucleoside transporters: from scavengers to novel therapeutic targets. Trends in pharmacological sciences *27*, 416-425.

Koc, A., Wheeler, L.J., Mathews, C.K., and Merrill, G.F. (2004). Hydroxyurea arrests DNA replication by a mechanism that preserves basal dNTP pools. The Journal of biological chemistry *279*, 223-230.

Kufe, D.W., Weichselbaum, R., Egan, E.M., Dahlberg, W., and Fram, R.J. (1984). Lethal effects of 1-beta-D-arabinofuranosylcytosine incorporation into deoxyribonucleic acid during ultraviolet repair. Molecular pharmacology *25*, 322-326.

Kumagai, A., Lee, J., Yoo, H.Y., and Dunphy, W.G. (2006). TopBP1 activates the ATR-ATRIP complex. Cell *124*, 943-955.

Kunos, C.A., Radivoyevitch, T., Pink, J., Chiu, S.M., Stefan, T., Jacobberger, J., and Kinsella, T.J. (2010). Ribonucleotide reductase inhibition enhances chemoradiosensitivity of human cervical cancers. Radiation research *174*, 574-581.

Kunos, C.A., Radivoyevitch, T., Waggoner, S., Debernardo, R., Zanotti, K., Resnick, K., Fusco, N., Adams, R., Redline, R., Faulhaber, P.*, et al.* (2013). Radiochemotherapy plus 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP, NSC #663249) in advanced-stage cervical and vaginal cancers. Gynecologic oncology *130*, 75-80.

Kunz, B.A., Kohalmi, S.E., Kunkel, T.A., Mathews, C.K., McIntosh, E.M., and Reidy, J.A. (1994). International Commission for Protection Against Environmental Mutagens and Carcinogens. Deoxyribonucleoside triphosphate levels: a critical factor in the maintenance of genetic stability. Mutat Res *318*, 1-64.

Lecona, E., Barrasa, J.I., Olmo, N., Llorente, B., Turnay, J., and Lizarbe, M.A. (2008). Upregulation of annexin A1 expression by butyrate in human colon adenocarcinoma cells: role of p53, NF-Y, and p38 mitogen-activated protein kinase. Molecular and cellular biology *28*, 4665-4674.

Lecona, E., and Fernandez-Capetillo, O. Replication stress and cancer: it takes two to tango. Experimental cell research *329*, 26-34.

Lecona, E., and Fernandez-Capetillo, O. (2014). Replication stress and cancer: it takes two to tango. Experimental cell research *329*, 26-34.

Lee, Y.D., and Elledge, S.J. (2006). Control of ribonucleotide reductase localization through an anchoring mechanism involving Wtm1. Genes & development *20*, 334-344.

Lee, Y.D., Wang, J., Stubbe, J., and Elledge, S.J. (2008). Dif1 is a DNA-damage-regulated facilitator of nuclear import for ribonucleotide reductase. Molecular cell *32*, 70-80.

Levin, V.A. (1992). The place of hydroxyurea in the treatment of primary brain tumors. Seminars in oncology *19*, 34-39.

Lewis, W.H., and Wright, J.A. (1974). Altered ribonucleotide reductase activity in mammalian tissue culture cells resistant to hydroxyurea. Biochemical and biophysical research communications *60*, 926-933.

Lin, Z.P., Belcourt, M.F., Cory, J.G., and Sartorelli, A.C. (2004). Stable suppression of the R2 subunit of ribonucleotide reductase by R2-targeted short interference RNA sensitizes p53(-/-) HCT-116 colon cancer cells to DNA-damaging agents and ribonucleotide reductase inhibitors. The Journal of biological chemistry *279*, 27030-27038.

Lindemalm, S., Liliemark, J., Juliusson, G., Larsson, R., and Albertioni, F. (2004). Cytotoxicity and pharmacokinetics of cladribine metabolite, 2-chloroadenine in patients with leukemia. Cancer letters *210*, 171-177.

Liu, C., Powell, K.A., Mundt, K., Wu, L., Carr, A.M., and Caspari, T. (2003). Cop9/signalosome subunits and Pcu4 regulate ribonucleotide reductase by both checkpoint-dependent and -independent mechanisms. Genes & development *17*, 1130-1140.

Liu, Q., Guntuku, S., Cui, X.S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A.*, et al.* (2000). Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes & development *14*, 1448-1459.

Liu, X., Lai, L., Wang, X., Xue, L., Leora, S., Wu, J., Hu, S., Zhang, K., Kuo, M.L., Zhou, L.*, et al.* (2011). Ribonucleotide reductase small subunit M2B prognoses better survival in colorectal cancer. Cancer research *71*, 3202-3213.

Liu, X., Zhou, B., Xue, L., Shih, J., Tye, K., Lin, W., Qi, C., Chu, P., Un, F., Wen, W.*, et al.* (2006). Metastasis-suppressing potential of ribonucleotide reductase small subunit p53R2 in human cancer cells. Clinical cancer research : an official journal of the American Association for Cancer Research *12*, 6337-6344.

Liu, X., Zhou, B., Xue, L., Shih, J., Tye, K., Qi, C., and Yen, Y. (2005). The ribonucleotide reductase subunit M2B subcellular localization and functional importance for DNA replication in physiological growth of KB cells. Biochemical pharmacology *70*, 1288-1297.

Lopez-Contreras, A.J., and Fernandez-Capetillo, O. The ATR barrier to replication-born DNA damage. DNA Repair (Amst) *9*, 1249-1255.

Lopez-Contreras, A.J., and Fernandez-Capetillo, O. (2010). The ATR barrier to replication-born DNA damage. DNA Repair (Amst) *9*, 1249-1255.

Lopez-Contreras, A.J., Gutierrez-Martinez, P., Specks, J., Rodrigo-Perez, S., and Fernandez-Capetillo, O. (2012). An extra allele of Chk1 limits oncogene-induced replicative stress and promotes transformation. The Journal of experimental medicine *209*, 455-461.

Lopez-Contreras, A.J., Specks, J., Barlow, J.H., Ambrogio, C., Desler, C., Vikingsson, S., Rodrigo-Perez, S., Green, H., Rasmussen, L.J., Murga, M.*, et al.* (2015). Increased Rrm2 gene dosage reduces fragile site breakage and prolongs survival of ATR mutant mice. Genes Dev *29*, 690-695.

Lou, H., Komata, M., Katou, Y., Guan, Z., Reis, C.C., Budd, M., Shirahige, K., and Campbell, J.L. (2008). Mrc1 and DNA polymerase epsilon function together in linking DNA replication and the S phase checkpoint. Molecular cell *32*, 106-117.

Lozano, G., and Elledge, S.J. (2000). p53 sends nucleotides to repair DNA. Nature *404*, 24-25.

Lu, A.G., Feng, H., Wang, P.X., Han, D.P., Chen, X.H., and Zheng, M.H. (2012). Emerging roles of the ribonucleotide reductase M2 in colorectal cancer and ultraviolet-induced DNA damage repair. World J Gastroenterol *18*, 4704-4713.

Lubelsky, Y., Reuven, N., and Shaul, Y. (2005). Autorepression of rfx1 gene expression: functional conservation from yeast to humans in response to DNA replication arrest. Molecular and cellular biology *25*, 10665-10673.

Mannava, S., Moparthy, K.C., Wheeler, L.J., Leonova, K.I., Wawrzyniak, J.A., Bianchi-Smiraglia, A., Berman, A.E., Flanagan, S., Shewach, D.S., Zeitouni, N.C.*, et al.* (2012).

Ribonucleotide reductase and thymidylate synthase or exogenous deoxyribonucleosides reduce DNA damage and senescence caused by C-MYC depletion. Aging (Albany NY) *4*, 917-922.

Mannava, S., Moparthy, K.C., Wheeler, L.J., Natarajan, V., Zucker, S.N., Fink, E.E., Im, M., Flanagan, S., Burhans, W.C., Zeitouni, N.C.*, et al.* (2013). Depletion of deoxyribonucleotide pools is an endogenous source of DNA damage in cells undergoing oncogene-induced senescence. Am J Pathol *182*, 142-151.

Mason, J.B. (2011). Unraveling the complex relationship between folate and cancer risk. BioFactors *37*, 253-260.

Mathews, C.K. (1985). Enzymatic channeling of DNA precursors. Basic Life Sci *31*, 47-66.
Mathews, C.K. (2006). DNA precursor metabolism and genomic stability. FASEB J *20*, 1300-1314.

Mathews, C.K., and Sinha, N.K. (1982). Are DNA precursors concentrated at replication sites? Proceedings of the National Academy of Sciences of the United States of America *79*, 302-306.

Matsumoto, M., Rey, D.A., and Cory, J.G. (1990). Effects of cytosine arabinoside and hydroxyurea on the synthesis of deoxyribonucleotides and DNA replication in L1210 cells. Adv Enzyme Regul *30*, 47-59.

Matsuoka, S., Ballif, B.A., Smogorzewska, A., McDonald, E.R., 3rd, Hurov, K.E., Luo, J., Bakalarski, C.E., Zhao, Z., Solimini, N., Lerenthal, Y.*, et al.* (2007). ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science *316*, 1160-1166.

Matsushita, S., Ikeda, R., Fukushige, T., Tajitsu, Y., Gunshin, K., Okumura, H., Ushiyama, M., Akiyama, S., Kawai, K., Takeda, Y.*, et al.* (2012). p53R2 is a prognostic factor of melanoma and regulates proliferation and chemosensitivity of melanoma cells. J Dermatol Sci *68*, 19-24.

McClarty, G.A., Chan, A.K., Engstrom, Y., Wright, J.A., and Thelander, L. (1987). Elevated expression of M1 and M2 components and drug-induced posttranscriptional modulation of ribonucleotide reductase in a hydroxyurea-resistant mouse cell line. Biochemistry *26*, 8004-8011.

Meurisse, J., Bacquin, A., Richet, N., Charbonnier, J.B., Ochsenbein, F., and Peyroche, A. (2014). Hug1 is an intrinsically disordered protein that inhibits ribonucleotide reductase activity by directly binding Rnr2 subunit. Nucleic acids research *42*, 13174-13185.

Minnihan, E.C., Nocera, D.G., and Stubbe, J. (2013). Reversible, long-range radical transfer in E. coli class Ia ribonucleotide reductase. Acc Chem Res *46*, 2524-2535.

Monasor, A., Murga, M., Lopez-Contreras, A.J., Navas, C., Gomez, G., Pisano, D.G., and Fernandez-Capetillo, O. (2013). INK4a/ARF limits the expansion of cells suffering from replication stress. Cell cycle *12*, 1948-1954.

Morikawa, T., Maeda, D., Kume, H., Homma, Y., and Fukayama, M. (2010). Ribonucleotide reductase M2 subunit is a novel diagnostic marker and a potential therapeutic target in bladder cancer. Histopathology *57*, 885-892.

Mosammaparast, N., and Pemberton, L.F. (2004). Karyopherins: from nuclear-transport mediators to nuclear-function regulators. Trends in cell biology *14*, 547-556.

Murga, M., Bunting, S., Montana, M.F., Soria, R., Mulero, F., Canamero, M., Lee, Y., McKinnon, P.J., Nussenzweig, A., and Fernandez-Capetillo, O. (2009). A mouse model of ATR-Seckel shows embryonic replicative stress and accelerated aging. Nature genetics *41*, 891-898.

Murga, M., Campaner, S., Lopez-Contreras, A.J., Toledo, L.I., Soria, R., Montana, M.F., D'Artista, L., Schleker, T., Guerra, C., Garcia, E.*, et al.* (2011). Exploiting oncogene-induced replicative stress for the selective killing of Myc-driven tumors. Nature structural & molecular biology *18*, 1331-1335.

Nakano, K., Balint, E., Ashcroft, M., and Vousden, K.H. (2000). A ribonucleotide reductase gene is a transcriptional target of p53 and p73. Oncogene *19*, 4283-4289.

Nathanson, D.A., Armijo, A.L., Tom, M., Li, Z., Dimitrova, E., Austin, W.R., Nomme, J., Campbell, D.O., Ta, L., Le, T.M.*, et al.* (2014). Co-targeting of convergent nucleotide biosynthetic pathways for leukemia eradication. The Journal of experimental medicine *211*, 473-486.

Nedelcheva, M.N., Roguev, A., Dolapchiev, L.B., Shevchenko, A., Taskov, H.B., Stewart, A.F., and Stoynov, S.S. (2005). Uncoupling of unwinding from DNA synthesis implies regulation of MCM helicase by Tof1/Mrc1/Csm3 checkpoint complex. J Mol Biol *347*, 509-521.

Nestoras, K., Mohammed, A.H., Schreurs, A.S., Fleck, O., Watson, A.T., Poitelea, M., O'Shea, C., Chahwan, C., Holmberg, C., Kragelund, B.B.*, et al.* Regulation of ribonucleotide reductase by Spd1 involves multiple mechanisms. Genes & development *24*, 1145-1159.

Nestoras, K., Mohammed, A.H., Schreurs, A.S., Fleck, O., Watson, A.T., Poitelea, M., O'Shea, C., Chahwan, C., Holmberg, C., Kragelund, B.B.*, et al.* (2010). Regulation of ribonucleotide reductase by Spd1 involves multiple mechanisms. Genes & development *24*, 1145-1159.

Nevins, J.R. (2001). The Rb/E2F pathway and cancer. Human molecular genetics *10*, 699-703.

Niida, H., Katsuno, Y., Sengoku, M., Shimada, M., Yukawa, M., Ikura, M., Ikura, T., Kohno, K., Shima, H., Suzuki, H.*, et al.* Essential role of Tip60-dependent recruitment of ribonucleotide reductase at DNA damage sites in DNA repair during G1 phase. Genes & development *24*, 333-338.

Niida, H., Katsuno, Y., Sengoku, M., Shimada, M., Yukawa, M., Ikura, M., Ikura, T., Kohno, K., Shima, H., Suzuki, H.*, et al.* (2010a). Essential role of Tip60-dependent recruitment of ribonucleotide reductase at DNA damage sites in DNA repair during G1 phase. Genes & development *24*, 333-338.

Niida, H., Shimada, M., Murakami, H., and Nakanishi, M. Mechanisms of dNTP supply that play an essential role in maintaining genome integrity in eukaryotic cells. Cancer science *101*, 2505-2509.

Niida, H., Shimada, M., Murakami, H., and Nakanishi, M. (2010b). Mechanisms of dNTP supply that play an essential role in maintaining genome integrity in eukaryotic cells. Cancer science *101*, 2505-2509.

Nordlund, P., and Reichard, P. (2006). Ribonucleotide reductases. Annu Rev Biochem *75*, 681-706.

O'Neill, T., Giarratani, L., Chen, P., Iyer, L., Lee, C.H., Bobiak, M., Kanai, F., Zhou, B.B., Chung, J.H., and Rathbun, G.A. (2002). Determination of substrate motifs for human Chk1 and hCds1/Chk2 by the oriented peptide library approach. The Journal of biological chemistry *277*, 16102-16115.

Oh, Y.K., and Park, T.G. (2009). siRNA delivery systems for cancer treatment. Advanced drug delivery reviews *61*, 850-862.

Okumura, H., Natsugoe, S., Yokomakura, N., Kita, Y., Matsumoto, M., Uchikado, Y., Setoyama, T., Owaki, T., Ishigami, S., and Aikou, T. (2006). Expression of p53R2 is related to prognosis in patients with esophageal squamous cell carcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research *12*, 3740-3745.

Paulsen, R.D., and Cimprich, K.A. (2007). The ATR pathway: fine-tuning the fork. DNA Repair (Amst) *6*, 953-966.

Perlstein, D.L., Ge, J., Ortigosa, A.D., Robblee, J.H., Zhang, Z., Huang, M., and Stubbe, J. (2005). The active form of the Saccharomyces cerevisiae ribonucleotide reductase small subunit is a heterodimer in vitro and in vivo. Biochemistry *44*, 15366-15377.

Pfisterer, J., Vergote, I., Du Bois, A., Eisenhauer, E., Ago, O., Ncic, C.T.G., and Eortc, G.C.G. (2005). Combination therapy with gemcitabine and carboplatin in recurrent ovarian cancer. International journal of gynecological cancer : official journal of the International Gynecological Cancer Society *15 Suppl 1*, 36-41.

Pitterle, D.M., Kim, Y.C., Jolicoeur, E.M., Cao, Y., O'Briant, K.C., and Bepler, G. (1999). Lung cancer and the human gene for ribonucleotide reductase subunit M1 (RRM1). Mamm Genome *10*, 916-922.

Plunkett, W., Huang, P., Xu, Y.Z., Heinemann, V., Grunewald, R., and Gandhi, V. (1995). Gemcitabine: metabolism, mechanisms of action, and self-potentiation. Seminars in oncology *22*, 3-10.

Poli, J., Tsaponina, O., Crabbe, L., Keszthelyi, A., Pantesco, V., Chabes, A., Lengronne, A., and Pasero, P. dNTP pools determine fork progression and origin usage under replication stress. EMBO J *31*, 883-894.

Poli, J., Tsaponina, O., Crabbe, L., Keszthelyi, A., Pantesco, V., Chabes, A., Lengronne, A., and Pasero, P. (2012). dNTP pools determine fork progression and origin usage under replication stress. The EMBO journal *31*, 883-894.

Pontarin, G., Fijolek, A., Pizzo, P., Ferraro, P., Rampazzo, C., Pozzan, T., Thelander, L., Reichard, P.A., and Bianchi, V. (2008). Ribonucleotide reduction is a cytosolic process in mammalian cells independently of DNA damage. Proceedings of the National Academy of Sciences of the United States of America *105*, 17801-17806.

Prem veer Reddy, G., and Pardee, A.B. (1980). Multienzyme complex for metabolic channeling in mammalian DNA replication. Proceedings of the National Academy of Sciences of the United States of America *77*, 3312-3316.

Rahman, M.A., Amin, A.R., Wang, X., Zuckerman, J.E., Choi, C.H., Zhou, B., Wang, D., Nannapaneni, S., Koenig, L., Chen, Z.*, et al.* (2012). Systemic delivery of siRNA nanoparticles targeting RRM2 suppresses head and neck tumor growth. Journal of controlled release : official journal of the Controlled Release Society *159*, 384-392.

Reichard, P. (1988). Interactions between deoxyribonucleotide and DNA synthesis. Annu Rev Biochem *57*, 349-374.

Reynolds, E.H. (2002). Folic acid, ageing, depression, and dementia. Bmj *324*, 1512-1515.

Rodriguez, C.I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A.F., and Dymecki, S.M. (2000). High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nature genetics *25*, 139-140.

Rofougaran, R., Vodnala, M., and Hofer, A. (2006). Enzymatically active mammalian ribonucleotide reductase exists primarily as an alpha6beta2 octamer. The Journal of biological chemistry *281*, 27705-27711.

Rossi, D.J., Bryder, D., Seita, J., Nussenzweig, A., Hoeijmakers, J., and Weissman, I.L. (2007). Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. Nature *447*, 725-729.

Ruiz, S., Lopez-Contreras, A.J., Gabut, M., Marion, R.M., Bau, S., Gutierrez-Martinez, P., Ramirez, O., Rodrigo-Perez, S., Iñigo Olalde, Li, H., Marques-Bonet, T., Serrano, M., Blasco, M.A., Batada, N.N. and Fernandez-Capetillo, O (2015). Limiting replication stress during somatic cell reprogramming reduces genomic instability in induced pluripotent stem cells. Nature Communications *In Press*.

Sabatinos, S.A., Green, M.D., and Forsburg, S.L. (2012). Continued DNA synthesis in replication checkpoint mutants leads to fork collapse. Molecular and cellular biology *32*, 4986-4997.

Sanchez-Romero, M.A., Molina, F., and Jimenez-Sanchez, A. (2010). Correlation between ribonucleoside-diphosphate reductase and three replication proteins in Escherichia coli. BMC Mol Biol *11*, 11.

Sandler, A., and Ettinger, D.S. (1999). Gemcitabine: single-agent and combination therapy in non-small cell lung cancer. The oncologist *4*, 241-251.

Sandoval, A., Consoli, U., and Plunkett, W. (1996). Fludarabine-mediated inhibition of nucleotide excision repair induces apoptosis in quiescent human lymphocytes. Clinical cancer research : an official journal of the American Association for Cancer Research *2*, 1731-1741.

Sanvisens, N., de Llanos, R., and Puig, S. (2013). Function and regulation of yeast ribonucleotide reductase: cell cycle, genotoxic stress, and iron bioavailability. Biomed J *36*, 51-58.

Sasaki, H., and Matsui, Y. (2008). Epigenetic events in mammalian germ-cell development: reprogramming and beyond. Nat Rev Genet *9*, 129-140.

Schaller, T., Pollpeter, D., Apolonia, L., Goujon, C., and Malim, M.H. (2014). Nuclear import of SAMHD1 is mediated by a classical karyopherin alpha/beta1 dependent pathway and confers sensitivity to VpxMAC induced ubiquitination and proteasomal degradation. Retrovirology *11*, 29.

Schulze, J., Lopez-Contreras, A.J., Uluckan, O., Grana-Castro, O., Fernandez-Capetillo, O., and Wagner, E.F. (2014). Fos-dependent induction of Chk1 protects osteoblasts from replication stress. Cell cycle *13*, 1980-1986.

Segurado, M., and Tercero, J.A. (2009). The S-phase checkpoint: targeting the replication fork. Biol Cell *101*, 617-627.

Shao, J., Liu, X., Zhu, L., and Yen, Y. (2013). Targeting ribonucleotide reductase for cancer therapy. Expert opinion on therapeutic targets *17*, 1423-1437.

Smith, M.L., and Seo, Y.R. (2002). p53 regulation of DNA excision repair pathways. Mutagenesis *17*, 149-156.

Sogo, J.M., Lopes, M., and Foiani, M. (2002). Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science *297*, 599-602.

Sommerhalter, M., Voegtli, W.C., Perlstein, D.L., Ge, J., Stubbe, J., and Rosenzweig, A.C. (2004). Structures of the yeast ribonucleotide reductase Rnr2 and Rnr4 homodimers. Biochemistry *43*, 7736-7742.

Sterkers, Y., Preudhomme, C., Lai, J.L., Demory, J.L., Caulier, M.T., Wattel, E., Bordessoule, D., Bauters, F., and Fenaux, P. (1998). Acute myeloid leukemia and myelodysplastic syndromes following essential thrombocythemia treated with hydroxyurea: high proportion of cases with 17p deletion. Blood *91*, 616-622.

Stubbe, J., and van der Donk, W.A. (1995). Ribonucleotide reductases: radical enzymes with suicidal tendencies. Chemistry & biology *2*, 793-801.

Takai, H., Tominaga, K., Motoyama, N., Minamishima, Y.A., Nagahama, H., Tsukiyama, T., Ikeda, K., Nakayama, K., and Nakanishi, M. (2000). Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice. Genes & development *14*, 1439-1447.

Tanaka, H., Arakawa, H., Yamaguchi, T., Shiraishi, K., Fukuda, S., Matsui, K., Takei, Y., and Nakamura, Y. (2000). A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. Nature *404*, 42-49.

Temperini, C., Innocenti, A., Scozzafava, A., and Supuran, C.T. (2006). N-hydroxyurea--a versatile zinc binding function in the design of metalloenzyme inhibitors. Bioorganic & medicinal chemistry letters *16*, 4316-4320.

Toledo, L.I., Murga, M., Gutierrez-Martinez, P., Soria, R., and Fernandez-Capetillo, O. (2008). ATR signaling can drive cells into senescence in the absence of DNA breaks. Genes & development *22*, 297-302.

Toledo, L.I., Murga, M., Zur, R., Soria, R., Rodriguez, A., Martinez, S., Oyarzabal, J., Pastor, J., Bischoff, J.R., and Fernandez-Capetillo, O. (2011). A cell-based screen identifies ATR inhibitors with synthetic lethal properties for cancer-associated mutations. Nature structural & molecular biology *18*, 721-727.

Tonin, P.N., Stallings, R.L., Carman, M.D., Bertino, J.R., Wright, J.A., Srinivasan, P.R., and Lewis, W.H. (1987). Chromosomal assignment of amplified genes in hydroxyurea-resistant hamster cells. Cytogenet Cell Genet *45*, 102-108.

Tourriere, H., and Pasero, P. (2007). Maintenance of fork integrity at damaged DNA and natural pause sites. DNA Repair (Amst) *6*, 900-913.

Toy, G., Austin, W.R., Liao, H.I., Cheng, D., Singh, A., Campbell, D.O., Ishikawa, T.O., Lehmann, L.W., Satyamurthy, N., Phelps, M.E.*, et al.* (2010). Requirement for deoxycytidine kinase in T and B lymphocyte development. Proceedings of the National Academy of Sciences of the United States of America *107*, 5551-5556.

Tsaponina, O., Barsoum, E., Astrom, S.U., and Chabes, A. (2011). Ixr1 is required for the expression of the ribonucleotide reductase Rnr1 and maintenance of dNTP pools. PLoS genetics *7*, e1002061.

Uhlin, U., and Eklund, H. (1994). Structure of ribonucleotide reductase protein R1. Nature *370*, 533-539.

Uramoto, H., Sugio, K., Oyama, T., Hanagiri, T., and Yasumoto, K. (2006). P53R2, p53 inducible ribonucleotide reductase gene, correlated with tumor progression of non-small cell lung cancer. Anticancer Res *26*, 983-988.

van der Watt, P.J., Maske, C.P., Hendricks, D.T., Parker, M.I., Denny, L., Govender, D., Birrer, M.J., and Leaner, V.D. (2009). The Karyopherin proteins, Crm1 and Karyopherin beta1, are overexpressed in cervical cancer and are critical for cancer cell survival and proliferation. Int J Cancer *124*, 1829-1840.

van der Watt, P.J., Ngarande, E., and Leaner, V.D. (2011). Overexpression of Kpnbeta1 and Kpnalpha2 importin proteins in cancer derives from deregulated E2F activity. PloS one *6*, e27723.

van der Watt, P.J., Stowell, C.L., and Leaner, V.D. (2013). The nuclear import receptor Kpnbeta1 and its potential as an anticancer therapeutic target. Critical reviews in eukaryotic gene expression *23*, 1-10.

Verlinden, L., Vanden Bempt, I., Eelen, G., Drijkoningen, M., Verlinden, I., Marchal, K., De Wolf-Peeters, C., Christiaens, M.R., Michiels, L., Bouillon, R.*, et al.* (2007). The E2F-regulated gene Chk1 is highly expressed in triple-negative estrogen receptor /progesterone receptor /HER-2 breast carcinomas. Cancer research *67*, 6574-6581.

Voegtli, W.C., Ge, J., Perlstein, D.L., Stubbe, J., and Rosenzweig, A.C. (2001). Structure of the yeast ribonucleotide reductase Y2Y4 heterodimer. Proceedings of the National Academy of Sciences of the United States of America *98*, 10073-10078.

Walling, J. (2006). From methotrexate to pemetrexed and beyond. A review of the pharmacodynamic and clinical properties of antifolates. Investigational new drugs *24*, 37-77.

Wang, L.M., Lu, F.F., Zhang, S.Y., Yao, R.Y., Xing, X.M., and Wei, Z.M. (2012). Overexpression of catalytic subunit M2 in patients with ovarian cancer. Chin Med J (Engl) *125*, 2151-2156.

Wang, X., Zou, L., Lu, T., Bao, S., Hurov, K.E., Hittelman, W.N., Elledge, S.J., and Li, L. (2006). Rad17 phosphorylation is required for claspin recruitment and Chk1 activation in response to replication stress. Molecular cell *23*, 331-341.

Warner, H.R. (1973). Properties of ribonucleoside diphosphate reductase in nucleotide-permeable cells. J Bacteriol *115*, 18-22.

Weinberg, G., Ullman, B., and Martin, D.W., Jr. (1981). Mutator phenotypes in mammalian cell mutants with distinct biochemical defects and abnormal deoxyribonucleoside triphosphate pools. Proceedings of the National Academy of Sciences of the United States of America *78*, 2447-2451.

Weinert, T. (1998). DNA damage and checkpoint pathways: molecular anatomy and interactions with repair. Cell *94*, 555-558.

Wright, J.A., Alam, T.G., McClarty, G.A., Tagger, A.Y., and Thelander, L. (1987). Altered expression of ribonucleotide reductase and role of M2 gene amplification in hydroxyurea-resistant hamster, mouse, rat, and human cell lines. Somatic cell and molecular genetics *13*, 155-165.

Wu, X., and Huang, M. (2008). Dif1 controls subcellular localization of ribonucleotide reductase by mediating nuclear import of the R2 subunit. Molecular and cellular biology *28*, 7156-7167.

Xie, M., Yen, Y., Owonikoko, T.K., Ramalingam, S.S., Khuri, F.R., Curran, W.J., Doetsch, P.W., and Deng, X. (2014). Bcl2 induces DNA replication stress by inhibiting ribonucleotide reductase. Cancer research *74*, 212-223.

Xu, X., Page, J.L., Surtees, J.A., Liu, H., Lagedrost, S., Lu, Y., Bronson, R., Alani, E., Nikitin, A.Y., and Weiss, R.S. (2008). Broad overexpression of ribonucleotide reductase genes in mice specifically induces lung neoplasms. Cancer Res *68*, 2652-2660.

Xu, Y.Z., Huang, P., and Plunkett, W. (1995). Functional compartmentation of dCTP pools. Preferential utilization of salvaged deoxycytidine for DNA repair in human lymphoblasts. The Journal of biological chemistry *270*, 631-637.

Yamaguchi, T., Matsuda, K., Sagiya, Y., Iwadate, M., Fujino, M.A., Nakamura, Y., and Arakawa, H. (2001). p53R2-dependent pathway for DNA synthesis in a p53-regulated cell cycle checkpoint. Cancer research *61*, 8256-8262.

Yanamoto, S., Kawasaki, G., Yamada, S., Yoshitomi, I., Yoshida, H., and Mizuno, A. (2009). Ribonucleotide reductase small subunit p53R2 promotes oral cancer invasion via the E-cadherin/beta-catenin pathway. Oral Oncol *45*, 521-525.

Yanamoto, S., Kawasaki, G., Yoshitomi, I., and Mizuno, A. (2003). Expression of p53R2, newly p53 target in oral normal epithelium, epithelial dysplasia and squamous cell carcinoma. Cancer letters *190*, 233-243.

Yang, X.H., and Zou, L. (2006). Recruitment of ATR-ATRIP, Rad17, and 9-1-1 complexes to DNA damage. Methods in enzymology *409*, 118-131.

Zeman, M.K., and Cimprich, K.A. Causes and consequences of replication stress. Nature cell biology *16*, 2-9.

Zeman, M.K., and Cimprich, K.A. (2014). Causes and consequences of replication stress. Nature cell biology *16*, 2-9.

Zhang, Y.W., Jones, T.L., Martin, S.E., Caplen, N.J., and Pommier, Y. (2009). Implication of checkpoint kinase-dependent up-regulation of ribonucleotide reductase R2 in DNA damage response. J Biol Chem *284*, 18085-18095.

Zhang, Z., Yang, K., Chen, C.C., Feser, J., and Huang, M. (2007). Role of the C terminus of the ribonucleotide reductase large subunit in enzyme regeneration and its inhibition by Sml1. Proceedings of the National Academy of Sciences of the United States of America *104*, 2217-2222.

Zhao, X., Chabes, A., Domkin, V., Thelander, L., and Rothstein, R. (2001). The ribonucleotide reductase inhibitor Sml1 is a new target of the Mec1/Rad53 kinase cascade during growth and in response to DNA damage. The EMBO journal *20*, 3544-3553.

Zhao, X., Georgieva, B., Chabes, A., Domkin, V., Ippel, J.H., Schleucher, J., Wijmenga, S., Thelander, L., and Rothstein, R. (2000). Mutational and structural analyses of the ribonucleotide reductase inhibitor Sml1 define its Rnr1 interaction domain whose inactivation allows suppression of mec1 and rad53 lethality. Molecular and cellular biology *20*, 9076-9083.

Zhao, X., Muller, E.G., and Rothstein, R. (1998). A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. Molecular cell *2*, 329-340.

Zhao, X., and Rothstein, R. (2002). The Dun1 checkpoint kinase phosphorylates and regulates the ribonucleotide reductase inhibitor Sml1. Proceedings of the National Academy of Sciences of the United States of America *99*, 3746-3751.

Zhou, B.B., and Elledge, S.J. (2000). The DNA damage response: putting checkpoints in perspective. Nature *408*, 433-439.

Zhu, L., Zhou, B., Chen, X., Jiang, H., Shao, J., and Yen, Y. (2009). Inhibitory mechanisms of heterocyclic carboxaldehyde thiosemicabazones for two forms of human ribonucleotide reductase. Biochemical pharmacology *78*, 1178-1185.

ANNEX